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(54) Title: METHOD OF INHIBITING CELL PROLIFERATION USING APOLIPOPROTEIN E

(57) Abstract

The present invention provides a use of ApoE for inhibition of proliferation of actively proliferating cells including tumor cells. This method comprises contacting the cells with an amount of Apolipoprotein E (ApoE) effective to inhibit cell proliferation.

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METHOD OF INHIBITING CELL PROLIFERATION USING APOLIPOPROTEIN E

Background of the Invention

This invention relates to the use of Apolipprotein E in a method of inhibiting cell proliferation.

Throughout this specification, various publications are referenced within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this applciation in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention disclosed and claimed herein.

In normal tissue, cell growth and DNA synthesis are closely controlled by a variety of regulatory factors operating on both positive and negative levels (Weinberg et al., 1989). As a normal cell develops into a solid tumor, it undergoes several changes (Folkman, 1989; Liotta, 1992). At the physiologic level, growth is stimulated, immunity is decreased, and new blood vessel formation is induced. This capacity to induce new blood vessels, i.e., angiogenesis and neovascularization, is characteristic of most malignant cells and is a prerequisite of solid tumor growth (D'Amore, 1988; Folkman, 1976). Moreover, new blood vessels penetrating the tumor are frequent sites for tumor cell entry into the circulation. Angiogenesis is also necessary for expansion of a metastatic colony (Aznavoorian, 1991). In addition to malignant cell growth, other diseases are

also characterized by abnormal neovascularization, including neovascular glaucoma, diabetic retinopathy, and rheumatoid arthritis (Folkman, et al., 1989).

Malignant cells produce many factors that stimulate endothelial cell proliferation and migration and allow new capillary beds to form within the tumor nodule (D'Amore, 1988; Shing, et al., 1985). A variety of agents have been suggested as potential modulators of cell proliferation, including heparin and heparin sulfate (Castellot, et al., 1987; Clowes, 1977), and growth factors and their inhibitors (Edelman, et al., 1992; Liu, 1990; Schweigerer, et al., 1987). Most of these factors also appear to be natural components of normal tissue. To date, the most studied growth factor has been basic fibroblast growth factor (bFGF), a member of the fibroblast growth factor family (Basilico and Moscatelli, 1992; Schweigerer, et al., 1987; Thomas and Gimenez-Gallego, 1986). bFGF is a strong heparin-binding molecule, present in virtually all tissues and having multiple mitogenic and angiogenic effects (Thomas and Gimenez-Gallego, 1986). In vascular endothelial cells, bFGF stimulates a number of functions involved in the formation of blood vessels and angiogenesis. bFGF is considered to be one of the most potent angiogenesis inducers both in vivo and in vitro (Folkman, 1976; Folkman and Klagsbrun, 1987). Recently it was shown that intravenous infusion of bFGF stimulated endothelial regeneration and SMC proliferation (Edelman, et al., 1992; Lindner and Reidy, 1991; Lindner, et al., 1990) after balloon-induced endothelial denudation. In these studies, it was confirmed that bFGF was both angiogenic and mitogenic for SMC in vivo and also demonstrated that these two effects are coupled. bFGF also binds to heparan sulfate proteoglycans (HSPG) of both the extracellular matrix (ECM) and basement membrane (Folkman, et al., 1988; Vlodavsky, et

al., 1987). Thus, it has been postulated that bFGF may play an important role in the pathogenesis of atherosclerotic vascular disease.

The role of HSPG in regulating cell growth and differentiation has been described (Burgess and Maciag, 1989; Klagsbrun and Baird, 1991; Ruoslahti and Yamaguchi, 1991). Many proteoglycans are constituents of the ECM or function as a low-affinity cell surface receptor for the interaction of growth factors, including bFGF and other heparin-binding growth factor molecules. The role of HSPG as binders of bFGF appears to protect bFGF from degradation, and is important in providing a matrix or cell surface bound reservoir of bFGF. Yayon, et al. (1991) have shown that bFGF binding to its high-affinity receptor requires prior binding either to the heparan sulfate side chains of a membrane HSPG or to free heparan sulfate (heparin) chains, and speculate that glycosaminoglycans may change the conformation of bFGF so that it acquires the ability to bind to its receptor. Binding of growth factors to proteoglycans have also been observed with several other growth factors that bind to heparin or heparan sulfate (Ruoslahti and Yamaguchi, 1991). Because proteoglycans are abundant and ubiquitous tissue components, they are likely to attract most of these growth factors and cytokines that have affinity for the glycosaminoglycan. It may be that growth factors and cytokines were meant to act on their target cells only over a short range, and that their immobilization at the cell surface and in ECM (through proteoglycan binding) accomplishes that goal (Ruoslahti and Yamaguchi, 1991).

Apolipoprotein E (ApoE) is a plasma protein having strong affinity for heparin and HSPG (Cardin, et al., 1988; Mahley, 1988; Mahley, et al., 1979; Weisgraber, et al., 1986). ApoE

participates in plasma lipoprotein metabolism thr ugh its high-affinity interaction with cell surface receptors including the low-density lipoprotein (LDL) and the more recently identified apoE receptor, LDL receptor-related protein (LRP) (Hertz, et al., 1988; Lund, et al., 1989; Yamada, et al., 1989; 1992). The domain of apoE responsible for binding to the LDL receptor has been identified (Dyer and Curtiss, 1991; Wilson, 1991). This domain is a 20-amino acid region comprised of residues 140-160 of the apoE molecule. It has long been known that binding of ApoE to the LDL receptor depended on its association with lipids (Innerarity, 1979). However, from results with synthetic peptides binding to the LDL receptor in vitro, one can assume there is direct binding of the peptide to the LDL receptor, or one could speculate that the LDL receptor is not the only binding site on the cell.

It has been shown that intravenous administration of ApoE into hyperlipidemic rabbits resulted in reduced plasma cholesterol levels (Mahley, et al., 1979; Yamada, et al., 1989). Recently (Yamada, et al., 1992), following a sustained intravenous administration of ApoE into Watanabe heritable hyperlipidemic rabbits, progression of atherosclerosis was significantly prevented. Administration of exogenous ApoE had affected both the number and size of atherosclerotic lesions in the aorta. However, in these experiments there was no significant difference in plasma cholesterol levels between ApoE-treated and nontreated control animals. Based on these and other experiments, the effect of ApoE on atherogenesis might neither be solely nor directly related to plasma cholesterol levels.

ApoE is ubiquitously synthesized in many tissues including liver, intestine, adrenal gland, kidney, lung, spleen, testes, ovary, and brain (Mahley, 1988). Recently, it has

be n found in both inflamed and non-inflamed synovial fluid (Terkeltaub, et al., 1991). ApoE can function in tissue repair by modulating lipid redistribution locally (Hui, et al., 1980; Mahley, 1988). However, it has also been observed that apoE is synthesized and secreted by a number of cells that do not necessarily participate in cholesterol homeostasis (Boyles, et al. 1989; Hui, et al., 1980).

In addition to its effect on lipoprotein metabolism, apoE also possesses a variety of functions that are unrelated to lipid transport (Mahley, 1988). A potent suppression of lymphocyte activation by mitogens and antigens by ApoE-bearing lipoproteins and ApoE polypeptides has been observed (Cardin, et al. 1988; Hui, et al., 1980). The present invention discloses the effect of ApoE on proliferation and migration of several cell types.

Summary f the Invention

A method of inhibiting actively proliferating cells is disclosed. This method comprises contacting the cells with Apolipoprotein E (ApoE) in an amount effective to inhibit cell proliferation.

A composition is provided comprising Apolipoprotein E in an amount effective to inhibit cell proliferation.

The invention additionally provides a method of treating a subject suffering from excessive cell proliferation which comprises contacting the excessively proliferating cells with an effective amount of Apolipoprotein E so as to inhibit the excessive cell proliferation.

Further, the present invention provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of an Apolipoprotein E in conjunction with a chemotherapeutic agent so as to inhibit proliferation of the tumor cells.

Additionally, the present invention provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of Apolipoprotein E in conjunction with an amount of irradiation so as to inhibit proliferation of the tumor cells.

Additionally, the invention provides a method of treating a subject suffering from a disorder involving increased neovascularization. This method comprises administering to the subject an amount of Apolipoprotein E effective to normalize neovascularization.

Brief Description of the Figures

Figure 1: The effect of heparin binding molecules on the incorporation of [^{3}H]thymidine into bovine aortic endothelial cell (BAEC) DNA was tested as described in Examples 1 and 2 using protocol M2, either in the presence of fetal calf serum (FCS) (1% and 2.5%) alone [control (ctr)], or with added basic fibroblast growth factor (bFGF). (All other samples were tested using 10 ng/ml). When indicated, 0.05 or 0.5 μM of met-apolipoprotein E (+E.05 or +E0.5, respectively) or recombinant thrombospondin (rTSP) 18 Kd (+T.05 or +T.5) were added to the wells containing FCS and bFGF. The mitogenesis assay was terminated after 42 hr.

Figure 2: The time course of the incorporation of [^{3}H]thymidine into BAEC DNA was obtained. DNA synthesis was tested on a newly-attached cell culture according to mitogenesis protocol M2 (see Example 1-Methods). Cells were plated in Dulbecco's modified Eagles's medium (DMEM)-containing bFGF (10 ng/ml) and 1% FCS alone (ctr) or together with 0.5 μM of the indicated molecules [(rTSP) 18 Kd, recombinant fibronectin (rFN) 33Kd, or met-apoE].

Figure 3: Incorporation of [^{3}H]thymidine into pre-attached BAEC culture was tested according to protocol M1, at either 0.5% FCS alone or together with bFGF (10 ng/ml). Met-apoE at the indicated concentrations was added to the cells 5 days after plating, and [^{3}H]thymidine incorporation was tested the next day after pulsing the cells for 5 hr and as indicated in protocol M1.

Figure 4: Incorporation of [^{3}H]thymidine into a pre-attached, dense culture of BAEC was studied in a preattached culture. Mitogenesis was tested according to protocol M1, using 0% or 0.5% FCS (0 and .5, respectively), together with

bFGF, 10 ng/ml (0F and .5F, respectively), r in combination with 0.5 μM met-apoE (0FE and .5FE, respectively). The mitogenesis assay was terminated after 40 hr.

Figure 5: Incorporation of [^{3}H]thymidine into BAEC culture was tested in the presence of bFGF (10 ng/ml) and one of two concentrations of FCS (as indicated in the figure) either alone (control), or together with met-apoE (0.5 μM), added to the newly plated culture at time zero (designated as 0-40), or at 15 hr or 22 hr after plating (designated as 15-40 and 22-40, respectively). [^{3}H]thymidine was also added at time zero to all wells as indicated in protocol M2, and the assay was terminated after 40 hr. Incorporation is expressed as a percent of control labelled with [^{3}H]thymidine for the same time in the absence of ApoE.

Figure 6: The effect of bFGF on inhibition by ApoE was studied. The proliferation assay was performed with culture of BAEC-1, as indicated in protocol P1, in the presence of 5% FCS either alone (ctr) or together with 20 ng/ml of bFGF. Where indicated, various concentrations of met-apoE were added 1 day after cell plating. The cell number was monitored as indicated in protocol P1.

Figure 7: Incorporation of [^{3}H]thymidine into bovine corneal endothelial cell (CBEC) culture was studied using various concentrations of FCS (ctr) as indicated (0%, 1%, or 2%). To this, growth factors were added either alone (bFGF [10 ng/ml] or EGF [50 ng/ml]), or together with met-apoE (E, 0.5 μM) or FN 33 Kd (FN, 0.5 μM). The mitogenesis assay was performed as described in protocol M2, and terminated after 44 hr.

Figure 8: The proliferation assay was performed with a culture of CBEC as indicated in protocol P2, in the presence of FCS and bFGF alone (control) at 5% and 10 ng/ml, respectively. When indicated, various concentrations of either met-apoE or FN 33 (rFN 33 Kd) were added to the cells at time zero. The average absorbancy of triplicate samples was calculated and expressed as percent of control.

Figure 9: The reversibility of ApoE inhibition was studied. CBEC culture in 1% FCS and 10 ng/ml bFGF was tested for [^{3}H]thymidine incorporation after 40 hr of labeling using protocol M2 (control). To parallel cultures, met-apoE was added at time zero at the indicated concentrations and either left for the entire labeling period (0-40) or for only 22 hr (0-22). For all tested cultures, media was replaced after 22 hours with the appropriate combination, including the starting concentrations of FCS, bFGF, and [^{3}H]thymidine.

Figure 10: Incorporation of [^{3}H]thymidine into culture of A2058 human melanoma cells was tested as described in protocol M2, and in the presence of 0.5% FCS and bFGF (10 ng/ml) either alone (ctr) or together with 0.5 μM and 1.5 μM met-apoE (0.5E and 1.5E, respectively), 0.5 μM TSP 18 Kd (0.5T18), 0.5 μM TSP 28 Kd (0.5T28), or 0.5 μM FN 33 Kd (0.5FN33). The assay was terminated after 32 hr.

Figure 11: Incorporation of [^{3}H]thymidine into a culture of human mammary tumor (MDA-435) cells was carried out according to protocol M2, and in the presence of 0.5% FCS and bFGF (10 ng/ml) alone (ctr) or with the addition of 0.5 μM met-apoE (E) or TSP 18Kd or with 75 μg/ml heparin (Hep). The assay was terminated after 42 hr.

Figur 12: Proliferation of smooth muscle cells (SMC) was performed as indicated in protocol P1, in the presence of either 0.5% or 5% FCS alone (ctr), or combined with bFGF (+F, 20 ng/ml). Where indicated, met-apoE (E) (4 μM) was added to the culture at time zero.

Figure 13: The incorporation of ^{35}S-methionine was examined as published (D. Blake 1990) with a slight modification. Briefly, CBEC cells were plated into 6-well tissue culture dishes in DMEM + 10% FCS at 10^5 cells/well. After 24 hr, cells were washed 3 times with PBS and the media was changed to DMEM minus methionine (for methionine depletion) either alone (ctr) or together with met-apoE (0.5 μm). After 1 hr, 0.25 μCi [^{35}S]methionine (1268 Ci/mmol, Amersham) was added, and the culture was incubated at 37°C/5% CO_2 for 6 hr. Cells were washed twice with phosphate buffered saline (PBS) containing 10 mM EDTA and 1 mM phenyl-methyl-sulfonyl and then suspended in the same buffer. The cells were lysed with 20 mM ammonium hydroxide (NH4OH), then aliquots of the cell lysate were precipitated with 5 volumes of 20% trichloracetic acid (TCA). After 10 minutes in ice the TCA solution was filtered on glass microfiber filters (GF/C, Whatman), then the filters were washed 3 times with 5% cold TCA and 1 time with 70% ethanol. The radioactivity of the TCA-insoluble [^{35}S]methionine-labeled protein was monitored by β counts.

Figure 14: Chemotaxis of BAEC to bFGF was carried out in a modified Boyden chamber as previously described (Taraboletti, 1990) using 5 μm pore size polycarbonate PVP-free nucleopore filters. Semiconfluent cells were trypsinized, washed with 10% FCS, allowed to equilibrate in 10% FCS-DMEM for 2 hours at room temperature while shaking, and then pelleted, and resuspended in medium containing 0.1% BSA. Cells were used immediately at a concentration of

10^6/ml and checked for migration either alone or together with the indicated concentration of met-apoE (E) toward a gradient of 0.1% BSA alone (ctr) or together with bFGF (33 ng/ml). Migrated cells in triplicate samples were monitored after 4.5 hr of incubation at 37°C, and are expressed as a percent of the migration toward 0.1% BSA (ctr).

Figure 15: This figure shows plasmid pTVR 590-4 which was deposited in E. coli W1485 under ATCC Accession No. 67360. Plasmid pTVR 590-4 is a good expressor of met-apoE under control of the $^{\lambda}P_L$ promoter as is described in Example 1. (E. coli W1485 is freely available from ATCC under Accession No. 12435.)

Figure 16: The construction of plasmid pE2-5 encoding ApoE containing amino acids 1-217 is described. Plasmid pTV 194-80 (disclosed in coassigned U.S. Patent No. 5,126,252, Figure 22) was digested with restriction enzymes BssHII and BglII. The large fragment was isolated and ligated to the synthetic linkers shown in the Figure. The resulting plasmid was designated pE2-5.

Figure 17: The construction of plasmid pTVR6-2 expressing ApoE having amino acids 1-217 is described. A 1200bp fragment containing the $^{\lambda}$ CI gene under control of a portion of the deoP1 promotor sequence was isolated from ClaI digestion of plasmid pFSAL-B27 (ATCC Accession No. 67071; also disclosed in European Patent Application Publication No. 303,972). The 1200bp fragment was then ligated to ClaI digested plasmid pE2-5 (Figure 16). The resulting plasmid, designated pTVR6-2, contains both the ApoE structural gene and the $^{\lambda}$ CI repressor gene, and is therefore an independent plasmid, not limited to use in a host containing the $^{\lambda}$ CI repressor but able to express the ApoE polypeptide fragment in a wide variety of hosts. It is

not presently known whether an additi nal N-terminal methionine is present. Plasmid pTVR6-2 was deposited in E. coli 4300 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland on July 26, 1993 under Accession No. 69364.

Figure 18: The effect of ApoE on proliferation of mouse endothelioma-END2 cells in culture was studied. The proliferation assay of END2 cells was performed as Example 7 using 20,000 cells per well and in the presence of 0.5% FCS, and the indicated concentrations of met-apoE.

Figure 19: The effect of ApoE on the proliferation of BAEC and CHO cells was studied. The proliferation of BAEC and CHO cells performed with 30,000 cells/ml in the presence of 0.5% FCS and the indicated concentrations of met-apoE as described in Example 2.

Figure 20: The effect of ApoE on the proliferation of BAEC and neuroblastoma cells was studied. The proliferation of BAEC and neuroblastoma N18TG2 was performed with 20,000 cells per ml in the presence of 0.5% FCS and the indicated concentrations of met-apoE (20A) and the ApoE-peptide 348 (20B) as described in Example 2.

Figure 21: The heat-stability of the anti-proliferative activity of ApoE was examined. The proliferation of BAEC cells (30,000 cells/ml) was tested in the presence of 0.5% FCS and the indicated concentrations of unheated or heated (100°C for 1hr) met-apoE as described in Example 2.

Figure 22: The effect of serum concentration on the anti-proliferative activity of met-apoE was studied. The proliferation of BAEC cells was measured at 0.5% and 2.5% FCS and in the presence of the indicated concentrations of

ApoE with ut th addition of exogenous growth factors. Met-apoE exhibits a lower degree of inhibition at the higher serum concentration as described in Example 2.

Figure 23: The effect of the ApoE designated apoE6-2 on the proliferation of END-2 cells was studied. The proliferation of END-2 was measured as indicated in Figure 18 in the presence of 0.5% FCS and the indicated amounts of the ApoE polypeptide fragment encoded by plasmid pTVR6-2 (Figure 17) as described in Example 8.

Figure 24: The effect of serum concentration on the anti- proliferative activity of an ApoE polypeptide was studied. The proliferation of BAEC cells was measured at 0.5% and 2.5% FCS and the indicated concentrations of the ApoE polypeptide apoE6-2 as described in Example 8.

Figure 25: The reversal of heparin activity by ApoE was studied. ApoE has a high affinity for heparin, and this property of ApoE was studied by the effect of met-apoE on a complex consisting of heparin, antithrombin III and thrombin. Addition of ApoE to the complex reverses the heparin activity resulting in inhibition of the antithrombin activity of heparin. A nonreactive short peptide designated peptide 185 was used as a negative control. A second negative control [ctrl (-)] did not include any ApoE. A positive control [ctrl (+)] did not contain heparin. The results indicate that at the ApoE concentration at which there is 50% inhibition of thrombin acrtivity, there is about a 3:1 ratio of heparin:ApoE, i.e. 2-3 molecules of heparin are bound by each molecule of ApoE.

Detailed Description of the Invention

The present invention provides a method of inhibiting actively proliferating cells comprising contacting actively proliferating cells with an amount of Apolipoprotein E (ApoE) effective to inhibit proliferation. Inhibition of proliferation means reduction of the rate of proliferation of the cells.

The cells may be smooth muscle cells, endothelial cells e.g. aortic or corneal endothelial cells, or tumor cells, e.g. human melanoma cells, mammary tumor cells, human sarcoma cells, or carcinoma cells. Other actively proliferating cell types known to those skilled in the art are also encompassed by the methods of the invention.

A composition for inhibiting the proliferation of actively proliferating cells comprising Apolipoprotein E and a suitable carrier is also provided.

Additionally, a method is provided of treating a subject suffering from excessive cell proliferation which comprises administering to the subject a amount of Apolipoprotein E effective to inhibit the excessive cell proliferation. Such a method may involve administration of the Apolipoprotein E in conjunction with other therapeutic means such as a chemotherapeutic agent or irradiation treatment, i.e. administration of ApoE prior to, during or after the other therapeutic means. Other therapeutic means for use in conjunction with Apolipoprotein E are known to those skilled in the art and are also encompassed by the methods of the invention.

A pharmaceutical composition is provided which comprises Apolipoprotein E in an amount effective to inhibit excessive

proliferation and a pharmaceutically acceptable carrier.

In one embodiment, excessive cell proliferation is a tumor.

The term "Apolipoprotein E" (ApoE) as used herein encompasses any polypeptide, regardless of source e.g. naturally occurring or recombinant which includes the sequence of naturally occurring apoE necessary for the biological activity of inhibiting proliferation of cells, and mutants whose sequence varies by one or more, typically less than ten amino acids provided that such mutants have the biological activity of inhibiting proliferation of cells.

Naturally occurring apoE may be obtained from plasma or serum by methods known to those skilled in the art and is available commercially e.g. Calbiochem cat. no. 178466.

Recombinant ApoE may be obtained from genetically engineered cells which produce recombinant ApoE. The cells may be of any strain in which a DNA sequence encoding recombinant ApoE has been introduced by recombinant DNA techniques so long as the cells are capable of expressing the DNA sequence and producing the recombinant ApoE polypeptide. The cells may contain the DNA sequence encoding the recombinant ApoE in a vector DNA molecule such as a plasmid which may be constructed by recombinant DNA techniques so that the sequence encoding the recombinant ApoE is incorporated at a suitable position in the vector. The cells are preferably bacterial cells or other unicellular organisms, but eucaryotic cells such as yeast, insect or mammalian cells may also be used to produce recombinant ApoE.

In one embodiment the ApoE is a mutant of recombinant apoE differing from the naturally occurring polypeptide by the

addition, deletion, or substitution of one or more non-essential amino acid residues, typically less than 10, provided that the resulting polypeptide retains the cell proliferation inhibitory activity of apoE. Persons skilled in the art can readily determine which amino acids residues may be added, deleted, or substituted (including with which amino acids such substitutions may be made) using established well known procedures, including, for example, conventional methods for the design and manufacture of DNA sequences coding for bacterial expression of mutants of the subject polypeptide, the modification of cDNA and genomic sequences by site-directed mutagenesis techniques, the construction of recombinant proteins and expression vectors, the bacterial expression of the polypeptides, and the determination of the biochemical activity of the polypeptides using conventional biochemical assays.

Examples of mutants of apoE are deletion mutants containing less than all the amino acid residues of naturally occurring apoE, substitution mutants wherein one or more residues are replaced by other residues, and addition mutants wherein one or more amino acids residues are added to the polypeptide. All such mutants share the cell proliferation inhibitory activity of naturally occurring apoE.

Polypeptides having substantially the same amino acid sequence as naturally occurring apolipoprotein E encompass the addition or deletion of fewer than four amino acids at the N-terminus of the amino acid sequence of the polypeptide. There may be additional substitutions and/or deletions in the sequence which do not eliminate the cell proliferation inhibiting biological activity of the polypeptide. Such substitutions and deletions are known to those skilled in the art. Substitutions may encompass up to about 10 residues in accordance with the homologous or

equivalent groups described by e.g. Lehninger, Biochemistry, 2nd ed. Worth Pub., N.Y. (1975); Creighton, Protein Structure, a Practical Approach, IRL Press at Oxford Univ. Press, Oxford, England (1989); and Dayhoff, Atlas of Protein Sequence and Structure 1972, National Biomedical Research Foundation, Maryland (1972).

In a particular embodiment, the ApoE is recombinant met-apoE, e.g. a recombinant polypeptide comprising the sequence of naturally occurring apoE with an additional methionine at the N-terminus.

Also encompassed by the term "Apolipoprotein E" are polypeptide fragments of recombinant ApoE and of naturally occurring apoE which exhibit the cell proliferation inhibitory activity of apoE. One example of such a fragment is a 15-mer fragment disclosed in U.S. Patent No. 5,177,189, issued January 5, 1993.

Additional examples of such polypeptide fragments have amino acids 1-217 or 1-185 of naturally occurring apoE. A particular embodiment of an ApoE polypeptide having amino acids 1-217 of naturally occurring apoE is encoded by plasmid pTVR6-2 (Figure 17) which was deposited in E. coli 4300 on July 26, 1993 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland under Accession No. 69364. Another fragment is the 22KD N-terminal ApoE polypeptide produced by thrombin digestion of naturally occurring apoE or recombinant met-apoE.

Similar ApoE polypeptides may be obtained by those skilled in the art from plasmids constructed on the basis of any of the above described plasmids and their use is encompassed by the claims defining the invention. Procedures for constructing such plasmids and obtaining such polypeptides

ar well kn wn to those skilled in the art and are described in numerous publications including Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, USA (1989).

In sum, the method of the present invention may be practiced with any ApoE having at least substantially the same cell proliferation inhibitory activity as naturally occurring apoE.

Further, the invention provides in vitro methods of inhibiting DNA synthesis of proliferating cells. This method comprises contacting the cells with ApoE in an amount effective to inhibit DNA synthesis. The invention also provides in vitro methods of inhibiting the chemotactic response of endothelial cells. This method comprises contacting cells with ApoE in an amount effective to inhibit the chemotactic response.

As used herein, chemotactic response means the migration of cells in response to a stimuli. The present invention provides a method whereby cells induced to migrate by exposure to a growth factor are inhibited by the contacting of ApoE with the migrating cells. The amount effective to inhibit a chemotactic response is any amount effective to inhibit the migration of cells stimulated in response to a growth factor.

It is herein disclosed that ApoE inhibits proliferation of various mammalian cells including aortic and corneal endothelial cells and mammary carcinoma cells, melanoma, and smooth muscle cells. As used herein, carcinoma refers to a malignant epithelial tumor. Furthermore, since ApoE inhibits aortic endothelial cells, it will likely inhibit neovascularization. Without neovascularization, i.e., the

f rmation of new blood vessels, cells cannot actively proliferate. In the case of tumor cells, inhibition or neovascularization results in inhibition of proliferation and thus interferes with tumor growth. Consequently, administration of ApoE is a novel treatment for the inhibition of the formation of new blood vessel growth (angiogenesis), with concommitant inhibition of tumor cell growth, and metastasis. Thus both indirectly by inhibition of angiogenesis, and directly, ApoE inhibits proliferation of a wide range of tumor cells including melanoma, sarcoma, lymphoma and leukemia cells.

It is herein disclosed that ApoE inhibits proliferation of smooth muscle cells. The formation and progression of plaques and metastatic tissue, due to abnormal neovascularization, is accompanied by the migration of smooth muscle cells to the site of the plaque or the metastatic tissue. Inhibition of proliferation of smooth muscle cells may interfere with the progression of formation of plaques and metastatic tissue.

Furthermore, abnormal neovascularization of smooth muscle cells may lead to the formation of a sarcoma. As used herein, sarcoma refers to a soft tissue tumor. Thus, treatment by ApoE for the inhibition of proliferating smooth muscle cells is also provided, thus constituting a treatment for the modulation of the formation and progression of plaques, metastatic tissue, and sarcoma tumors.

It is additionally provided that ApoE may be used therapeutically as an inhibitor of blood vessel formation for treatment of subjects suffering from disorders characterized by abnormal neovascularization. Abnormal neovascularization means the increased and enhanced ability to form blood vessels. Examples of abnormal

ne vascularization include such disorders as neovascular glaucoma, diabetic retinopathy, rheumatoid arthritis (Folkman, et al., 1989) and hemangioma.

In the mammalian body, cells may be actively proliferating because they are tumor cells or because they are being stimulated by endogenous or naturally occurring growth factors, or serum factors, or for other reasons.

Preferably, the ApoE is administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carrier encompasses any of the standard pharmaceutical carriers such as sterile solution, tablets, coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stensic acid, talc, vegetable fats or olis, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives and other ingredients.

Compositions comprising such carriers are formulated by well known conventional methods. However, a composition comprising ApoE in an amount effective to inhibit proliferation of actively proliferating cells was previously unknown.

In the method of this invention, the administration of the ApoE-containing composition may be effected by any of the well known methods, including but not limited to, oral, intravenous, intramuscular, and subcutaneous administration.

In the practice of the method of this invention, the amount of Apolipoprotein E incorporated in the composition may vary widely. The amount of ApoE effective to inhibit cell proliferation is 0.1mg - 1g ApoE. The precise amount and the frequency of administration of the dose will readily be

determined by one skilled in the art, based on the characteristics of the formulation, body weight and condition of the subject, tumor size, route and frequency of administration, and the characteristics of the particular Apolipoprotein E to be used.

EXAMPLES

This invention is illustrated by the Examples which follow. These Examples present specific embodiments and are set forth to aid in an understanding of the invention but are not intended to and should not be construed to limit in any way the invention as set forth in the claims which follow.

Example 1. Methods and Materials

A. Methods and Materials used to measure cellular growth

Cellular growth was evaluated under different growth conditions and by various methods, including DNA synthesis (mitogenesis) by measuring incorporation of ^{3}H-thymidine into DNA, and proliferation, by directly quantitating cell numbers by their associated enzymatic activity.

Polypeptides

Recombinant met-apoE contains the amino acid sequence of the human ApoE3 isoform with an additional N-terminal methionine (Vogel, et al. PNAS 1985) and was produced as described in section B below.

Plasmatic apoE was kindly provided by S. Eiesenberg (Laboratory of Lipids, Hadassa Medical School, Ein Kerem, Jerusalem). It was isolated from plasma lipoproteins derived from healthy human volunteers homozygous for the E3 isoform and as described earlier (Rall S.C. et al , Methods In Enzymology 128:273 (1986)).

The ApoE designated ApoE6-2, spanning amino acids 1-217 of apoE was produced as described in Example 8.

The ApoE designated peptide 348, a tandem dimeric peptide spanning amino acids 141-155 of apoE was prepared as described in Example 2.

The ApoE produced by thrombin digestion of apoE which removes the C-terminus, may be produced as described in Example 8.

Recombinant TSP18 is an 18 Kd polypeptide fragment, and recombinant TSP28 a 28 Kd polypeptide fragment, both of which contain the heparin binding domain comprising amino acids 1-174 and 1-242, respectively of human thrombospondin.

rFN33 is a recombinant 33 kD polypeptide fragment of the cell binding domain of human fibronectin comprising amino acids 1329 - 1722, but deleted of amino acids 1600-1689. The plasmid designated pFN 137-2 which encodes for the 33 kD polypeptide fragment has been fully described in co-assigned patent application U.S. Serial No. 291,951, filed December 29, 1988, and has been deposited in Escherichia coli strain A4255 under ATCC Accession No. 67910.

The recombinant polypeptides listed above were stored lyophilized at -70°C.

Met-apoE was reconstituted by first dissolving in distilled water at a concentration of 2mg/ml followed by the addition of 0.1 volumes of 10X PBS. rFN33 was dissolved in distilled water at a concentration of 3.5-5 mg/ml. rTSP 18 and 28 were first dissolved in distilled water at 0.5 mg/ml, desalted on a PD10 column (Pharmacia #170851-01) that was equilibrated with 10 mM sodiumbicarbonate pH 9.5, and then eluted with the same buffer. One tenth the volume of a solution of 10X PBS was then added. Polypeptide samples were stored in small aliquots at -20°C and used within one month. Heparin (Sodium, injection USP, 1000 U/m10, 6.25 mg/ml) was supplied by Lilly.

Cell Lines and Reagents

Bovine aortic endothelial cells (BAEC), were kindly provided by Dr. E. Gallin (AFRY, Bethesda, MD), and were used at

passages 5-10. BAEC culture was routinely maintained in 1 w glucose DMEM, containing 10% FCS, 4 mM glutamine, 0.5 mg/ml ascorbic acid and 500 u/ml penicillin and 500 u/ml streptomycin. (Biofluids Inc., Rockville, MD).

Bovine corneal endothelial cells (CBEC), were kindly provided by Dr. D. Blake. (Maharry Medical College, Nashville, TN), and used at passages 2-8. CBEC culture was routinely maintained in low glucose DMEM containing 10% FCS, 4 mM glutamine, 500 u/ml penicillin, 500 u/ml streptomycin, and 2.5μg/ml Fungison (BioFluids, Rockville, MD, 20850). The media was changed every 2-3 days.

The human melanoma cell line A2058 cells (Todaro, et al. Proc. Natal. Acad. Sci. U.S.A., 77, 5258-5262, 1980) and the human mammary tumor cell line MDA-MB 435 (Coillean, et al., (1978) In Vitro 14, 911-915), were maintained in high glucose DMEM, containing 10% FCS, 4mM glutamine and 500 u/ml penicillin and 500 u/ml streptomycin purchased from Biofluids, Inc., Rockville, MD 20850, USA. The human smooth muscle fibroblasts (SMC) were obtained from Dr. Philip Browning of the National Cancer Institute, National Institute of Health, Bethesda, Maryland. The cells were cultured in RPMI medium containing 10% FCS, 4mM glutamine, 500 u/ml penicillin, and 500 u/ml streptomycin (purchased from BioFluids).

Mouse endothelioma cells (END2) (Williams et al., Cell 57:1053 (1989)) expressing the polyoma middle-T antigen were provided by I. Voldavsky (Hadassa Medical School, Ein Kerem, Jerusalem). The END2 cells were routinely maintained in low glucose DMEM containing 10% FCS, 4mM glutamine, 500U/ml each of penicillin and streptomycin. END2 cells were normally used at 70-80% confluency after 5-8 days culture. Medium was replaced every 3-4 days. Medium components were obtained

from Kibbutz Beit Haemek, Israel.

Chinese hamster ovary cells (CHO) and neuroblastoma N18TG2 cells (Z. Vogel, Weizmann Institute) were routinely maintained in high glucose DMEM containing 10% FCS, 2mM glutamine, and 500U/ml each of penicillin and streptomycin. Medium components were obtained from Kibbutz Beit Haemek, Israel.

Assays

Both ApoE and bFGF are heparin-binding molecules, exhibiting high affinity for heparin and HSPG both on the cell surface and the ECM. The effect of ApoE on bFGF-stimulated growth was examined in several cell types in two separate systems: mitogenesis-by following the incorporation of [^{3}H]thymidine into DNA; and proliferation-by measuring the actual number of cells in the culture.

1. Inhibition of Mitogenesis

Protocol M1: Pre-attached, dense culture.

Cells were seeded at 10^5/well in 0.5 ml of medium supplemented with 10% FCS in the inner well of a 24-well tissue culture plate. After 3 days, the cells were washed with PBS and fed with 0.5 ml of medium containing 0.5% FCS and remained in this medium for 48 hr (starvation conditions). Serum-free medium containing the tested growth effectors was then added to the wells and 22 hours later the cells were labeled for 5 hours with [methyl-^{3}H]thymidine (86.1 ci/mmol, NEN; 2.5 μCi/well). To terminate the mitogenesis assay, the cells were washed twice with 1 ml PBS, fixed with 0.3 ml solution of methanol/acetic acid (3:1), washed twice with 0.5 ml ethanol (80%), and air-

dried. Th cells were extracted from the wells by incubation with 300 µl trypsin/EDTA (1 hr at 37°C and 30 min at room temperature), and by the addition of 100 µl 1% SDS. The radioactivity of the extracted material was measured in a scintillation counter.

Protocol M2: Newly-attached, medium-dense culture.

Confluent monolayers of cells in T-150 cm^2 flasks were washed once in PBS, and then incubated in 0.5% FCS-containing medium for 48 hours (starvation conditions). Cells were trypsinized, washed in 10% FCS-containing medium, resuspended in medium containing 0.1% BSA, and seeded in 24-well plates at 2 x 10^4 cells/well in the presence of various concentrations of FCS, growth effectors, and [^{3}H]thymidine (at the concentration indicated in protocol M1). At different time points (indicated in the figure legends), mitogenesis was terminated as described in protocol M1.

2. Inhibition of Proliferation

Protocol P1: 10^5 cells were plated into 35 mm culture dishes, in 10% FCS-containing medium, allowed to attach for 24 hours, and then refed with media containing 5% FCS and the tested growth effectors. After 72 hours, cells were detached from the plates by trypsinization, and their number monitored by a Coulter counter.

Protocol P2: This was carried out using the cell titer 96TM nonradioactive cell assay (Promega #G4000) based on the methods described in Denizot et al., J. Immunol. Meth. 89:271 (1986). 5-30x10^3 cells were plated into each well of a 96-well culture dish in medium containing 5% FCS together with the indicated concentrations of growth effectors. After 72 hours, 15 µl of dye solution was added to ach well

and the plates were incubated for an additional 4 hours. Then 100 µl of solubilization solution was added and after 24 hours, the amount of dye retained by the well was examined by recording absorbency at 570 nm using an ELISA plate reader.

3. The END2 hemangioma model

END2 cells, grown as described above, were harvested by trypsinization at mid-confluency after 5 days in culture. The cells were then suspended in DMEM containing 10% FCS, centrifuged, respended in basal DMEM without serum at $6x10^6$ cells/ml (or as otherwise indicated) and kept on ice. The cells were then diluted 1:1 with an extracellular matrix composition (Matrigel, H. Kleinman, Dental Institute, National Institutes of Health). Aliquots of 0.1ml were injected into the hind leg of female Balb/C mice (20-25g). On the ninth day, the mice were sacrificed and tumor development in the injected leg was observed. The tumor appeared as a hematomatous lump of purplish color the size of which varied in correlation with the number of cells initially injected. It was found that while a full size tumor developed after injection of 10^6 cells, the size was markedly smaller when 10^5 cells were injected, and only very small tumors developed following injection with $3x10^4$ cells. Therefore, the experiments were routinely performed with $3x10^5$ cells/mouse.

4. Thrombin activity

Thrombin activity may be measured by the hydrolysis of a chromogenic substrate resulting in release of a colored compound essentially as described by Lotenberg (BBA, 142:556 (1983)). Briefly, thrombin is able to cleave the synthetic substrat Tos-Gly-Pro-Arg-paranitroaniline resulting in the

release of paranitroaniline (PNA) whose concentration may be determined by absorbance at 405nm.

B. Production of ApoE

I. Host-Vector System For Expression of Recombinant ApoE

The preferred host-vector system used for production of met-ApoE is E. coli strain W1485 (ATCC No. 12435) harboring plasmid pTVR 590-4; the host-vector system has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland under ATCC Accession No. 67360.

The construction of plasmid pTVR 590-4 which is described below has been fully described in co-assigned copending patent application, EPO Publication No. 303,972 (see Figure 15).

Plasmid pTVR 590-4 contains the following elements:

a) Origin of replication.

b) The Amp^R gene in counter clockwise orientation.

c) In clockwise orientation and in 5′ to 3′ order, a truncated deo P1 promoter sequence and the lambda cI^{857} temperature-sensitive repressor coding sequence.

d) In counterclockwise orientation and in 5′ to 3′ order, the lambda promoter, the beta lactamase promoter-ribosomal binding site, the coding sequence for ApoE and the T_1T_2 transcription termination sequences.

This plasmid is a high level expressor of ApoE under the control of the strong leftward promoter of bacteriophage

lambda (P_L) which is thermoinducibly controlled by the constitutively expressed cI^{ts857} temperature-sensitive repressor also situated on the plasmid. Production of ApoE from this plasmid takes place upon heat- induction at 42°C.

This is a so-called "host independent" expression system since the plasmid can thermoinducibly produce met-ApoE independent of prior insertion of the lambda cI^{857} gene into the host E. coli chromosome. This plasmid can therefore be used to transform a wide variety of host bacterial cells. The host described, E. coli W1485 is a prototrophic wild-type strain of E. coli freely obtainable from the ATCC under Accession No. 12435.

II. Growth of E. coli W1485 harboring plasmid pTVR 590-4 and production of a bacterial cake containing ApoE

The following description of the fermentation of E. coli W1485 harboring plasmid pTVR 590-4 is a preferred embodiment for production of a cell cake containing ApoE.

1. Seed Flask Development

The contents of frozen vials containing E. coli ATCC No. 12435/pTVR 590-4 are used to inoculate seed flasks containing the following medium:

K_2HPO_4	9 g
KH_2PO_4	1 g
NaCl	5 g
$MgSO_4.7H20$	0.2 g
NH_4Cl	1 g
$FeNH_4$ citrate	0.01 g
Trace elements solution	1 ml
Biotin	0.5 mg
Glucose	5 g

Ampicillin, sodium salt	0.1 g
Deionized water	1 L

Trace elements stock solution:

$MnSO_4 .H_2O$	1 g
$ZnSO_4 .7H_2O$	2.78 g
$CoCl_2.6H_2O$	2 g
$Na_2MoO_4 .2H_2O$	2 g
$CaCl_2 .2H_2O$	3 g
$CuSO_4 .5H_2O$	1.85 g
H_3BO_3	0.5 g
Concentrated HCl	100 mL
Deionized Water	900 mL

Glucose and ampicillin are added from sterile concentrated stock solutions after autoclaving the other components of the medium. The cultures are incubated at 30°C overnight on a rotary shaker at 250 rpm, and reach an OD_{660} of 3.5-5.0.

2. Seed Fermenter

The contents of the seed flask are used to inoculate a 50 L seed fermenter containing 25-30 L of the following production medium, which contains per liter:

K_2HPO_4	8 g
KH_2PO_4	2 g
Sodium citrate	2 g
NH_4Cl	2 g
$FeNH_4$ citrate	0.02 g
$CaCl_2.2H_2O$	0.04 g
K_2SO_4	0.6 g
Trace elements solution (as in Section 1)	3 mL
Antifoam (Silicolapse 5000)	2 mL

Added after sterilization (per liter of medium)

$MgSO_4 .7H_2O$		0.4 g
Sodium ampicillin		0.1 g
Glucose		40-60 g
NH_3 (25-28% in water)	approx.	40 mL

Glucose is added batchwise at inoculation; ammonia is automatically added for pH control (set point pH = 7.0) during growth.

The culture is cultivated at 30°C for 15-20 hours in order to achieve growth; the OD_{660} generally reaches 20-30 during this time. This is equivalent to a dry cell weight (DCW) of 7.5-12 g/L.

3. Production Fermenter

The contents of the seed fermenter are used to inoculate a 750 L (nominal volume) fermenter containing about 360 L production medium as described for seed fermenter, but excluding ampicillin. The culture is cultivated at 30°C until an OD_{660} of 10 is obtained. Induction of ApoE expression is then achieved by raising the fermenter temperature to 42°C. At induction, the following are added to the fermenter:

DL-methionine	0.6 g per L of medium
Sodium acetate	5 g per L of medium

The sodium acetate (0.1% - 1%) is added to protect cells from the "toxic effect" caused by the ApoE analog.

The fermenter temperature is maintained at 42°C for three hours, at which time the cells are harvested. Th OD_{660} of

the cell suspension at harvest is generally 16-20, the volume is 400-430 L and the DCW is 5.0-6.5 g/L.

4. Harvest of Cells

The cell suspension is centrifuged at 14,000 rpm (16,000 g) in a CEPA 101 tubular bowl centrifuge at a feed rate of 250L/hr, and a cell cake weighing about 10 Kg is produced and saved. Alternatively, the cell suspension is centrifuged in a Westfalia CSA-19 continuous centrifuge at 500 L/hr. The sludge is either disrupted immediately or frozen.

In both cases, the supernatant contains no detectable ApoE as measured by SDS-polyacrylamide gel electrophoresis.

III. Purification of Recombinant ApoE

The following method is suitable for scale-up for industrial application and yields very pure ApoE. The general scheme of the downstream process (Scheme 1) consists of steps A through G as follows:

A CELL DISRUPTION IN PRESENCE OF MAGNESIUM IONS.
B EXTRACTION OF CELL PELLET WITH TRITON®.
C 100K ULTRAFILTRATION.
D DEAE CHROMATOGRAPHY
E Q SEPHAROSE CHROMATOGRAPHY
F CM SEPHAROSE CHROMATOGRAPHY
G 100K ULTRAFILTRATION - TRITON® REMOVAL.

The following detailed example of the steps in purification of ApoE was performed on 3 Kg cell cake. In addition we have successfully processed a 15 Kg cell cake using the

methods described below with only minor modifications involving scale-up in the size or the equipment used.

Steps A through D were performed on 2 batches of bacterial cake, each weighing 1.5 Kg. After step D, the two batches were combined and processed as one batch through steps E to G. Steps A, B, C were performed at 4°C - 10°C, except where otherwise indicated. All other activities were performed at room temperature.

A. CELL DISRUPTION IN PRESENCE OF MAGNESIUM IONS

1.5 Kg of wet cell cake was suspended in 6 L of buffer A which consists of 50 mM tris/HCl, 30 mM $MgCl_2$, 0.25% beta hydroxybutyrate sodium salt, pH=7.5. (The beta hydroxybutyrate was added as a protease inhibitor) This was then homogenized using a Kinematica homogenizer yielding 7.5 L of homogenate. Disruption was then performed using a Dynomill KDL bead mill disrupter (Willy A. Bachofen, Basel) at 5 L/hr (in two cycles). Three-fold dilution of the resulting suspension using buffer A yielded a volume of 22.5 L. This lysate contained about 6 g ApoE, i.e. about 4 g ApoE per Kg of original bacterial cake.

Centrifugation was then performed in a continuous CEPA-41 tubular bowl centrifuge, (Carl Padberg, Lahr/Schwarzwald) with a feed rate of 9 L/hr at 20,000 rpm (17,000 g). The pellet, weighing approximately 700 g and containing insoluble ApoE was saved and the supernatant was discarded. (Note that the ApoE is insoluble due to the presence of Mg^{++} ions.)

B. EXTRACTION OF CELL PELLET WITH TRITON^R

Six liters (1:10) of extraction buffer were added to the

pell t. (Extraction buffer: 50 mM tris/HCl, 20 mM EDTA, 0.3% Triton®, pH adjusted to 8.0 with HCl). Suspension was achieved using a homogenizer (Kinematica) at low speed. Then another 6 L extraction buffer was added (giving a final pellet:buffer ratio of 1:20) and the pH was adjusted to 4.5 with 1 N NaOH. The resulting 12 L suspension was incubated for 10 minutes at room temperature with stirring.

After incubation, the suspension was centrifuged on the CEPA 41 Centrifuge at a feed rate of 20 L/hr. The pellet weighing about 450 g was discarded and the supernatant solution containing ApoE was titrated to pH=7.5 with 1 N NaOH and saved.

Note: Triton® is present in all following steps and is removed in step G.

C. 100 K ULTRAFILTRATION

The purpose of this step is to remove low molecular weight contaminants by ultrafiltration/dialysis.

A Millipore Pellicon ultrafiltration system using one 100 K cassette type PTHK was utilized to concentrate the supernatant of the previous step (about 12 L) to about 2 L. The feed pressure was 20 psig and the filtrate flow rate was 20 L/hr. The dialysis buffer was 50 mM tris HCl, 10 mM EDTA and 0.1% Triton®, pH=7.5. The 2 L retentate containing about 2-3 mg ApoE /ml was kept cool with ice.

The retentate was dialyzed using the recirculating mode of the Pellicon ultrafiltration system until a filtrate conductivity equivalent to that of the dialysis buffer was obtained; this was the criterion used throughout the purification for termination of dialysis.

D. DEAE CHROMATOGRAPHY

The purpose of this step is to separate the ApoE from contaminants such as proteins and other cellular materials.

In this step a 1.6 L DEAE Sepharose Fast Flow column (Pharmacia) was used. The flow rate was 10 column volumes/hr. The capacity of the column under these conditions was determined to be 4 mg ApoE/ml. The column was first equilibrated with DEAE equilibration buffer: 20 mM tris/HCl, 1 mM EDTA, 0.5% Triton®, pH=7.5.

The retentate solution from the previous step (about 3 L) was then loaded on the column and washed with 3 column volumes (CV) of equilibration buffer. The first elution was performed using 3 CV of equilibration buffer containing 120 mM NaCl. Fractions were collected and the progress of the run was monitored by continuously following the absorbance of the eluate at 280 nm. The fractions were analyzed by SDS polyacrylamide gel electrophoresis stained by Coomassie Blue and the trailing edge of the peak (3.1 CV) was saved.

The second elution was performed using the equilibration buffer containing 150 mM NaCl. Fractions were collected and analyzed by SDS gel electrophoresis and most of the peak (3.9 CV) was saved. Endotoxins were measured by the Limulus Amebocyte Lysate (LAL) assay described in U.S. Pharmacopeia (U.S.P.) XXI, 1165-1166 (1985). The level of endotoxin was 3 μg per mg ApoE.

Concentration and dialysis after DEAE-Sepharose

The fractions indicated from the first and second eluates were pooled and dialyzed using the Pellicon ultrafiltration system, with one 100K cassette; the dialysis buffer was 20

mM tris/HCl, 1 mM EDTA, 0.1% Triton^R, pH=7.5. The sample was concentrated to 2 L (about 2-3 mg ApoE/ml) and dialyzed.

E. Q-SEPHAROSE (QS) CHROMATOGRAPHY

The purpose of this step is to separate active from inactive ApoE and to further remove endotoxins.

In this step a 1.6 L QS Fast Flow Column (Pharmacia) was used; the column capacity under these conditions was about 7 mg ApoE/ml and the flow rate was about 10 CV/hr.

The QS equilibration buffer was 20 mM tris/HCl, 1 mM EDTA, 0.2% Triton^R, pH=7.8. After equilibration, the retentate solutions from two batches of the previous step were combined and loaded on to the column, i.e. a total volume of about 5 L of buffer containing about 5 g ApoE. The column was then washed with 2.8 CV of equilibration buffer. The first elution was performed with 3 CV of equilibration buffer containing 20 mM NaCl and the second elution was performed with about 5.5 CV of equilibration buffer containing 40 mM NaCl. Fractions were collected, monitored and analyzed as described above, and 2.0 CV were combined and saved. The level of endotoxin was measured by the LAL assay and was now less than 250 pg/mg ApoE.

Two subsequent elutions using buffer containing 70 mM NaCl and 350 mM NaCl respectively eluted the inert ApoE.

Concentration and dialysis after Q-Sepharose

The QS-derived saved pooled fractions were concentrated and dialyzed by ultrafiltration through a Millipore Pellicon Ultrafiltration system using one 100K cassette.

The dialysis buffer was 10 mM tris/HCl, 1 mM EDTA, 0.1% Triton^R, pH=7.5. The sample was dialyzed using the recirculating mode whilst maintaining the ApoE concentration at 2-3 mg/ml. The final retentate volume was about 500 ml.

F. CM-SEPHAROSE CHROMATOGRAPHY

The purpose of this step is to further remove endotoxins and to lower the concentration of Triton^R to 0.05%.

In this step a 120 ml CM-Sepharose Fast Flow (Pharmacia) column was used. The equilibration buffer was 20 mM Na acetate, 1 mM EDTA, 0.2% Triton^R, pH=4.8. After equilibration, the retentate solution from the previous step was loaded on to the CM-Sepharose column. The capacity of the column was 10 mg ApoE/ml and the flow rate was 10 CV/hr.

The column was then washed with the following solutions: 4 CV of equilibration buffer followed by 5 CV of equilibration buffer containing 70 mM NaCl followed by 2 CV of 20 mM Na acetate, 1 mM EDTA, 0.05% Triton^R, 70 mM NaCl pH=4.8. The eluate from the loading and washing steps was discarded.

The column was then eluted. The eluent was 8 CV of 20 mM Na acetate, 1 mM EDTA, 0.05% Triton^R, 300 mM NaCl, pH=5.0. The progress of the elution was monitored by continuously following the absorbance of the eluate at 280 nm. (Two different base lines are used during the elution: one is the high U.V. absorbance buffer containing 0.2% Triton, the other is the low U.V. absorbance buffer containing 0.05% Triton. The use of a sensitivity scale of about 1.0 OD allows both buffers to appear on the chart column, the low at the foot and the high at about 0.5 OD.)

The sampl containing ApoE was immediately titrated to pH 7.8 and saved. The endotoxin level in this sample was below 50 pg per mg ApoE as measured by the LAL assay.

G. 100K ULTRAFILTRATION - TRITON^R REMOVAL

The purpose of this step is to remove the Triton^R.

This step was carried out at 4°C using the Millipore Pellicon Ultrafiltration System, containing one 100K cassette, pre-washed with 0.5 M NaOH overnight. The flow rate was 9-12 L/hr and the inlet/pressure was 5-10 psig. (This low flow rate is used to prevent aggregation of the ApoE as the Triton^R is being removed.) The ApoE sample from the previous step (960 ml containing about 600 mg ApoE) was diluted to 0.5 mg/ml with 10 mM $NaHCO_3$ buffer pH=7.7.

The sample was then treated in the ultrafiltration system and the following conditions were applied throughout this Triton^R removal step:

a) The Triton^R concentration must be lower than 0.02% i.e. the Triton^R concentration must be below its critical micelle concentration in order to achieve effective Triton^R removal across the 100K membrane.

b) The ApoE must not be diluted below 0.5 mg/ml or dissociation of the ApoE molecule will occur and it may cross the 100 K membrane.

c) The ApoE must not be concentrated above 1.5 mg/ml or aggregation of the ApoE molecules may occur.

The dialysis buffer used in the ultrafiltration system was 10 mM $NaHCO_3$, 150 mM NaCl, pH=7.8.

After concentration and dilution steps in accordance with the above conditions, the dialysis was performed at constant volume and constant flow rate and the dialysis was completed when the absorbance at 280 nm of the filtrate was 0.01 units. (Triton^R solution absorbs at 280 nm and an absorbance of 0.01 is equivalent to 0.0005% Triton^R.) The total volume of final retentate was 770 ml and the total volume of the filtrate was 9.5 L.

The solution containing the ApoE was then filtered (0.2 micron filter) and stored at -70°C in 80 ml glass bottles.

Overall Yield:

0.3 g of highly purified met-apoE were recovered from 3 Kg of bacterial cake. The ApoE, approximately 97% pure, was in the same aggregation state as plasmatic apoE when tested under the same conditions of gel permeation analysis. The ApoE sample contained less then 50 pg of endotoxins/mg protein.

Lyophilization

If the ApoE is to be lyophilized the dialysis buffer in the Triton^R removal step is 2 mM $NaHCO_3$ pH=7.8, 1mM cystein/mg apoE,, and after lyophilization the samples of ApoE are stored at -20°C.

After lyophilization, the ApoE can be redissolved and it retains its normal biological activity. The lyophilized ApoE is very stable for at least 5 years.

Example 2. Effect of ApoE on bovine aortic endothelial cell cultures

A. Effect of ApoE on incorporation of ^{3}H thymidine

Addition of bFGF to a freshly plated 48 hr serum-starved culture of bovine aortic endothelial cells (BAEC) stimulated the incorporation of [^{3}H]thymidine by several-fold compared to the control culture, both at 1% and 2.5% FCS, as shown in Figure 1. This stimulation of DNA synthesis is strongly inhibited by addition of ApoE to the culture, and was both dose- and serum-dependent. At 1% FCS, inhibition by 0.05 μM and 0.5 μM ApoE was approximately 85% and 98%, respectively. However, at 2.5% FCS, the inhibition was lower, i.e., in the range of 55% and 85%, respectively.

Addition of another heparin-binding molecule such as the recombinant TSP fragment (rTSP 18) resulted in much less inhibition of DNA synthesis at 1% FCS. At 2.5% FCS, the inhibition by ApoE and rTSP was identical.

B. Effect of ApoE on time course of thymidine incorporation

The effect of ApoE on the time-course of [^{3}H]thymidine incorporation in a freshly plated culture of BAEC in the presence of both bFGF and 1% FCS is demonstrated in Figure 2. [^{3}H]thymidine incorporation is time-dependent, reaching maximum capacity around 42 hrs after cessation of cell starvation. In the presence of 0.5 μM ApoE, incorporation of [^{3}H]thymidine was reduced by more than 95%. the residual activity (2-3%) of [^{3}H]thymidine incorporation had also reached a maximum after 42 hrs. In parallel cultures, 0.5 μM rTSP18 or rFN33 gave either 30% inhibition or no inhibition, respectively.

C. Effect of various concentrations of ApoE

The effect of various concentrations of ApoE on [^{3}H]thymidine incorporation of pre-attached cultures of BAEC was measured. The example in Figure 3 demonstrates that the inhibition obtained by 5 nM ApoE in the presence of 0.5% FCS and bFGF is approximately 45%, while in the presence of FCS alone, the inhibition by the same amount of ApoE is 10-fold lower (~ 4%). It should be noted that throughout the ongoing experiments, the stimulation of cell growth by exogenously added bFGF was variable. Generally, when cells display high basal activity of [^{3}H]thymidine incorporation at low serum concentrations (e.g., 0-0.5%), the cells are not further stimulated by added bFGF.

D. Effect of ApoE on growth factor dependent proliferation

Pre-attached cultures of BAEC plated at high density (10^5/well) demonstrate a very high level of [^{3}H]thymidine incorporation at both 0% and 0.5% FCS (as shown in Figure 4). The addition of bFGF to the cultures did not show any substantial stimulation of the [^{3}H]thymidine incorporation. However, only cultures containing the exogenous bFGF displayed a remarkable inhibition (~ 65%) by added ApoE. When ApoE was added to the culture containing only 0.5% FCS, the inhibitory effect of ApoE was many fold lower (~ 5%), i.e. ApoE inhibits the growth factor dependent proliferation of these cells.

E. Effect of time of addition of ApoE to bovine aortic endothelial cells

Newly attached BAEC cultures were allowed to grow in the presence of bFGF and either 0.5% or 1% FCS. The addition of ApoE at time zero together with bFGF caused the maximal

effect of inhibition. The addition of ApoE 15 or 22 hr later caused much less inhibition at both serum concentrations. The results are shown in Figure 5. Thus, the first 15 hr of growth are most effective for ApoE inhibition. After 15 hr, ApoE was considerably less effective in reducing DNA synthesis in these cultures. It should be noted that the cells became refractory to ApoE at a time prior to initiation of DNA synthesis based on the time course presented in Figure 2. Therefore, ApoE probably inhibits entry into S-phase but does not significantly arrest DNA synthesis once it is initiated.

The effect of ApoE on the proliferation of bovine aortic endothelial cells was determined in the presence of 5% FCS. Following 3 days of growth, the number of cells was examined by trypsinization and direct counting. When cells are plated in the absence of bFGF, the total increase in cell number was 10-15%, and there was no effect on cell number upon addition of ApoE. However, when bFGF was added to parallel cultures, the number of cells was increased by 2.5-fold. The addition of ApoE together with bFGF inhibited growth in a dose-dependent manner. At ApoE concentrations of 0.5 μM, 2.5 μM, and 5 μM, cell growth inhibition was approximately 10%, 40%, and 50%, respectively. These proliferation results are consistent with the thymidine incorporation results in Example 2D.

The effect of ApoE on the proliferation of vascular endothelial cells from bovine aorta (BAEC) was further studied without added exogenous bFGF. The results demonstrate the inhibition by ApoE of proliferation at 0.5% with the IC_{50} around 0.15μM. When heated at 100°C for 60 minutes, more than 95% of the antiproliferative activity of ApoE is lost, indicating the dependence of the inhibitory activity on the native form of the ApoE molecule (Figure

21). A similar degree of inhibition of proliferation of BAEC was also observed with apoE isolated from human plasma.

The specificity of the inhibition by ApoE was further demonstrated using peptide 348, a tandem dimeric peptide fragment of apoE derived from the LDL-receptor binding region and the strong heparin binding consensus sequence spanning amino acids 141-155 $(LRKLRKRLLRDADDL)_2$ (Dyer et al., J. Biol. Chem. 266:15009 (1991) and disclosed in U.S. Patent No. 5,177,189, issued January 5. 1993. The peptide, kindly provided by H. C. Krutzsch (Laboratory of Pathology, NCI, NIH) was synthesized by the standard solid phase method of Merrifield and purified as described earlier (Guo et al., PNAS 89:3040 (1992)).

Thus, the proliferation of BAEC cells is inhibited by met-apoE with an IC_{50} of 0.1-0.3μM (Figure 20A), and by peptide 348 with an IC_{50} of peptide 348 of 10-15μM (Figure 20B).

The inhibition of proliferation by met-apoE indicates selectivity for cell type and seems to favor vascular endothelial cells, since BAEC cells are more sensitive to inhibition than CHO cells (Figure 19) and neuroblastoma cells (Figure 20).

Moreover, the inhibition of proliferation of endothelial cells in the absence of exogenous bFGF is also observed at both low and high serum concentrations (Figure 22), thus indicating that the anti-proliferative activity of ApoE is effectively achieved in the presence of the endogenous naturally occurring growth factors normally present in serum. Furthermore, the observation of inhibition of serum dependent proliferation in the presence of other growth factors such as EGF (Figure 7) which are not heparin dependent, suggests that there are other mechanisms of

inhibition of proliferation by ApoE.

In another experiment, recombinant met-apoE was compared with plasmatic apoE in the BAEC cell proliferation assay at 0.5% serum concentration. The degree of inhibition was similar except for a slightly higher degree of inhibition exhibited by recombinant met-apoE.

In a further experiment, the effect in the BAEC cell proliferation assay of prior treatment of purified met-apoE with guanidine chloride (GuCl) was tested. The results showed that at 0.5% FCS, the GuCl treatment caused a slight increase in the inhibitory activity of met-apoE, while at 5% FCS, GuCl treatment induced a large increase in the degree of inhibition.

Example 3. Effect of ApoE on Bovine Cornea endothelial Cells

A. Effect of ApoE where CBEC are growth factor stimulated

The effect of ApoE on the mitogenesis and proliferation of a second type of endothelial dells, derived from bovine corneal endothelial cells (CBEC), was examined. The results are demonstrated in Figures 7 and 8, respectively. The results in Figure 7 describe the incorporation of [^{3}H]thymidine into CBEC, as was measured on a newly attached culture following 2 days serum starvation. At 0% FCS, the incorporation of [^{3}H]thymidine is relatively low. The incorporation is simulated several-fold when bFGF was added to the culture. The addition of met-apoE (at 0.5 μM) had dramatically reduced this bFGF-dependent DNA synthesis yielding inhibition of more than 90%. When cells are allowed to grow in the presence of 1% or 2% FCS, their initial growth was much greater and they were not stimulated by the addition of bFGF to the serum. With increasing serum concentrations, the ApoE-induced inhibition was reduced to about 50% and 35% at 1% and 2% FCS, respectively. Surprisingly, a similar mode of inhibition by ApoE was obtained with EGF-stimulated CBEC culture which is considered to be a non-heparin-binding growth factor. Here again, ApoE inhibited (>90%) the EGF-dependent growth, and to much less extent, the serum-dependent growth (45% and 25% at 1% and 2.5% FCS, respectively).

Based on the above results, ApoE will inhibit corneal endothelial cells from other mammalian (including human) sources, especially when the cells are actively proliferating.

B. Effect of ApoE when CBEC are serum stimulated

Higher concentrations of ApoE inhibit serum-stimulated proliferation in CBEC culture. The effect of met-apoE on CBEC proliferation was measured and is shown in Figure 8. CBEC were tested for proliferation at 5% FCS and in the presence of bFGF. The proliferation by bFGF was inhibited in a dose-dependent manner, with an IC_{50} ~1.5 μM. It is not clear whether the inhibition of proliferation in this case is dependent on the presence of bFGF. However, it is clear that ApoE inhibits proliferation of actively proliferating corneal endothelial cells.

C. Reversibility of ApoE inhibition

The inhibition of [^{3}H]thymidine incorporation by ApoE of CBEC culture is reversible, as shown in Figure 9. This reversibility is dependent upon the amount and time at which ApoE is given to the culture. When the newly attached CBEC cultures, in the presence of 1% FCS and bFGF, are maintained for the entire length of the mitogenesis experiment (40 hr) at 1.0 and 1.5 μM met-apoE, inhibition was 30% and 50%, respectively. However, when ApoE was given only for the first 22 hr, the inhibition by 1.0 and 1.5 μM ApoE following 40 hr incorporation, was much lower, e.g., in the order of 5% and 15%, respectively.

D. The effect of ApoE on Protein Synthesis of CBEC

Protein synthesis in CBEC was measured following a 1 hour starvation for methionine, and administration of S^{35}-methionine (6 hours), as shown in Figure 13. When met-apoE (0.5 μM) was added to a parallel culture, the incorporation of S^{35}-methionine into protein was identical to the control without ApoE. For both, the incorporation was approximately

60%. Thus, addition of ApoE has no direct effect on the synthesis of proteins.

Example 4. Effect of ApoE on Melanoma Cells

DNA synthesis in human A2058H melanoma cells was tested in a newly attached culture and in the presence of 0.5% FCS and bFGF, and following the addition of met-apoE, rTSP18 (rTSP18) and rFN33, as shown in Figure 10. Addition of ApoE at 0.5 and 1.0 μM caused inhibition of approximately 40% and 75%, respectively. No such inhibition was observed with the other molecules added in parallel. Indeed, addition of 0.5 μM of either TSP or FN stimulated [^{3}H]thymidine incorporation by 2-2.5-fold.

Example 5. Effect of ApoE on Carcinoma Cells

DNA synthesis in the human mammary carcinoma (MDA) cells was measured in a newly attached culture in 0.5% FCS and bFGF, as shown in Figure 11. Addition of 0.5 μM met-apoE or rTSP18 inhibited [^{3}H]thymidine incorporation by approximately 60% and 45%, respectively. Inhibition of DNA synthesis by heparin was lower (in the range of 30%).

Example 6. Effect of ApoE on Smooth Muscle Cells

The effect of ApoE on the proliferation of human smooth muscle cells (SMC) was tested at 0.5% and 5% FCS. In the presence of 0.5% FCS and a high concentration of met-apoE (4 μM), a strong inhibition of both serum and bFGF-dependent proliferation of SMC was obtained (approximately 80% inhibition in both cases). When serum concentration was increased to 5% the inhibition obtained by the same concentration of met-apoE was lower (approximately 30% for both serum and bFGF-dependent proliferation). The results are shown in Figure 12. Thus ApoE inhibits proliferation of actively proliferating human smooth muscle cells.

Example 7. Effect of ApoE on Mouse Endothelioma Cells

The antiproliferative and antiangiogenic activity of ApoE was further demonstrated in vitro and in vivo using mouse endothelioma cells END2 (Williams, Cell 1989). END2 are polyoma middle T-antigen transformed mouse endothelial cells, which develop spindle shaped cells in vitro and vascular tumors and hemangiomas in vivo. These cells have been used in vivo as a model system for studying angiogenesis. Cell proliferation was assayed as described in Example 1 according to protocol P1. Met-apoE inhibited the proliferation of END2 cells in culture with an IC_{50} of about 0.25μM at 0.5% FCS (Figure 18). Inhibition of proliferation by another ApoE is described in Example 8.

In addition, it was also demonstrated that ApoE inhibits the development of angiogenic lesions and hemangiomas in vivo in the endothelioma model. Thus, intravenous (i.v.) administration of met-apoE at a concentration of 0.4 mg/mouse, daily, for 8 days, resulted in a 40-60% reduction in the size of the hemangioma compared to the non-ApoE treated control.

Groups of ten mice were injected with the Matrigel suspension of END2 cells described above. The groups were then treated either with 0.2ml saline (control group) or 0.2ml saline containing 2mg/ml met-apoE (experimental group). The animals were sacrificed on the ninth day and the tumors were scored on a scale of 0-3 (0=no tumor). The results are shown in Table 1.

Table 1

Group	n[1]	Score[2]
Control	10	2.75±0.42
Experimental	10	1.7±0.82

[1] n=number of mice

[2] score: mean±standard deviation

The results were found to be significant ($P<0.005$) according to the Wilcoxon Rank Sum Test.

Based on the above results, ApoE will be effective in treating hemangiomas in humans.

Example 8. APO E Fragments

As mentioned earlier, ApoE has a specific ability to bind to lipoprotein particles for directing their removal from the plasma, "reverse cholesterol homeostasis", and for mediating receptor dependent endocytosis to the liver. Due to the high numbers of lipoprotein particles contained in plasma, ApoE, upon delivery in small amounts to the site of a tumor, will bind to lipoprotein particles and will be unavailable to function as an anti-proliferative agent. Therefore, full length ApoE (e.g. met-apoE) in the presence of serum or plasma is able to inhibit proliferation of cells only upon delivery in large amounts to the tumor.

This deficiency may be overcome by cleavage of the apoE sequence to remove the lipid binding domain present at the carboxy terminus. Upon cleavage of apoE with a proteolytic enzyme and elimination of the carboxyl end lipophilic domain, the remaining amino terminal fragment no longer has normal affinity for binding to lipoprotein particles but still readily binds to heparin and heparan sulfate proteoglycans. Therefore smaller amounts of ApoE will suffice to obtain inhibition of cell proliferation.

In a particular embodiment, such an ApoE fragment may be produced from apoE containing the complete sequence of naturally occurring apoE by cleavage of the carboxyl terminus with a proteolytic enzyme generating, for example, a 22 kD fragment containing the amino terminus (Thuren, 1992). One example of a proteolytic enzyme that may be used is thrombin. Upon digestion of recombinant met-apoE by thrombin , one 22 kD and one 10 kD fragment may be obtained. The 22 kD fragment, containing the first 191 amino terminal residues, has the binding sites for manganese heparan

sulfate and h parin and the low density lipoprotein (LDL) receptors, but has reduced ability to interact with natural lipoprotein particles.

An improved method of obtaining an increased amount of the free form of ApoE in the plasma and the reduction of its sequestration by lipid particles, is by production of an ApoE fragment deleted of the major lipid binding domain of apoE from the carboxyl terminus by means of recombinant DNA techniques.

In a specific embodiment, an ApoE polypeptide fragment, ApoE6-2, spanning amino acids 1-217 of apoE is encoded and expressed by plasmid pTVR6-2 (Figure 17). This 28KD MW ApoE was also tested for its effect on cell proliferation. The purification of this ApoE is similar to the purification of met-apoE as described in Example 1 with minor modifications, one of which was ultrafiltration with a 50K instead of 100K cutoff membrane. The purified polypeptide was then treated with 6M urea and dialyzed to remove the urea.

ApoE6-2 inhibited proliferation of BAEC culture with an IC_{50} around 0.3 μM at both 0.5% and 2.5% FCS respectively (Figure 24). Thus, in contrast to met-apoE, which displays a substantial reduction in activity at higher serum concentrations, ApoE6-2 is much less affected by increasing serum concentration.

This ApoE was also found to inhibit the proliferation of END-2 cells. The proliferation of END-2 was measured as indicated in Figure 18 in the presence of 0.5% FCS and the indicated amounts of ApoE6-2.

Based on these results, this and similar ApoE fragments will

provide high antiproliferative and antiangiogenic activity in vivo (where serum concentration is 100%).

Example 9. Reversal of antithrombin activity of heparin by ApoE

In this example, the effect of ApoE on the activity of heparin in a heparin-antithrombinIII-thrombin complex was demonstrated. Heparin activates antithrombinIII which in turn inhibits thrombin activity. Sequestration of heparin from this complex by ApoE results in the inactivation of antithrombinIII and a concomitant increase in thrombin activity. The effect of ApoE is dose dependent (Figure 25). The stoichiometry of the reaction indicates that ApoE displays high affinity for heparin, and that one mole of ApoE can bind 2-3 moles of heparin.

Thrombin activity was assayed as described in Example 1.

To be more specific, in a total volume of 140μl, antithrombin (50μl at 2.9U/ml, Sigma), heparin (15 μl, Eli Lilly 14KD, 0.4 USP/ml; 2μM final concentration in the reaction) and met-apoE (15μl of the indicated dilutions of a 60μM solution) were preincubated for 30 seconds. Thrombin (10μl, 20U/ml, Sigma) was then added and the reaction mix further incubated for an additional 30 seconds folowing which the chromogenic substrate (2μM, Sigma) was added. The results are shown in Figure 25. Under the reaction conditions used, no decrease in thrombin activity by heparin was observed until the met-apoE was diluted 64 fold. 50% inhibition of thrombin activity was achieved at approximately 1:100 dilution of ApoE (0.6μM) Therefore one molecule of ApoE can bind about 2-3 molecules of heparin.

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What is claimed is:

1. A method for inhibiting proliferation of actively proliferating cells which comprises contacting actively proliferating cells with an amount of Apolipoprotein E effective to inhibit proliferation.

2. The method of claim 1 wherein the cells are human smooth muscle cells.

3. The method of claim 1 wherein the cells are endothelial cells.

4. The method of claim 3 wherein the endothelial cells are aortic endothelial cells.

5. The method of claim 3 wherein the endothelial cells are corneal endothelial cells.

6. The method of claim 1 wherein the cells are tumor cells.

7. The method of claim 6 wherein the tumor cells are human melanoma cells.

8. The method of claim 6 wherein the cells are human mammary tumor cells.

9. The method of claim 6 wherein the cells are human sarcoma cells.

10. The method of claim 6 wherein the cells are carcinoma cells.

11. A composition for inhibiting the proliferation of

actively prolif rating cells comprising Apolipoprotein E and a suitable carrier.

12. A method of treating a subject suffering from excessive cell proliferation which comprises administering to the subject an amount of Apolipoprotein E effective to inhibit the excessive cell proliferation.

13. The method of claim 12, wherein the Apolipoprotein E is administered in conjunction with a chemotherapeutic agent.

14. The method of claim 12, wherein the Apolipoprotein E is administered in conjunction with irradiation treatment.

15. The method of claim 12 wherein the excessive cell proliferation comprises a tumor.

16. The method of claim 15 wherein the tumor cells are human melanoma cells.

17. The method of claim 15 wherein the tumor cells are human mammary tumor cells.

18. The method of claim 15 wherein the tumor cells are human sarcoma cells.

19. A method according to claim 12 wherein the excessive cell proliferation comprises abnormal neovascularization.

20. The method of claim 19, wherein the abnormal neovascularization is neovascular glaucoma.

21. The method of claim 19, wherein th abn rmal neovascularization is diabetic retinopathy.

22. The method of claim 19 wherein the abnormal neovascularization is present in rheumatoid arthritis.

23. The method of claim 19 wherein the abnormal neovascularization is a hemangioma.

24. The method of claim 12 wherein the Apolipoprotein E is administered intravenously.

25. The method of claim 12 wherein the Apolipoprotein E is administered subcutaneously.

26. The method of claim 12 wherein the Apolipoprotein E is administered intramuscularly.

27. A pharmaceutical composition comprising Apolipoprotein E in an amount effective to inhibit excessive proliferation and a pharmaceutically acceptable carrier.

28. The composition of claim 27 wherein the excessive cell proliferation is a tumor.

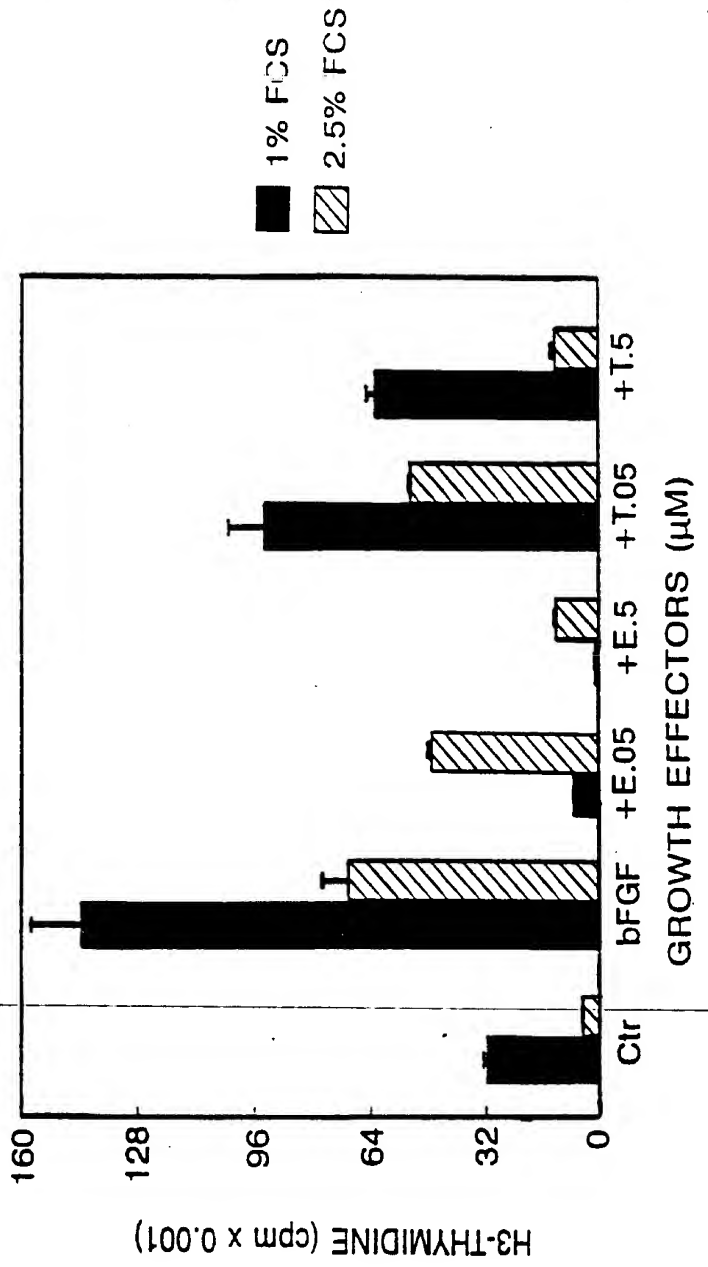
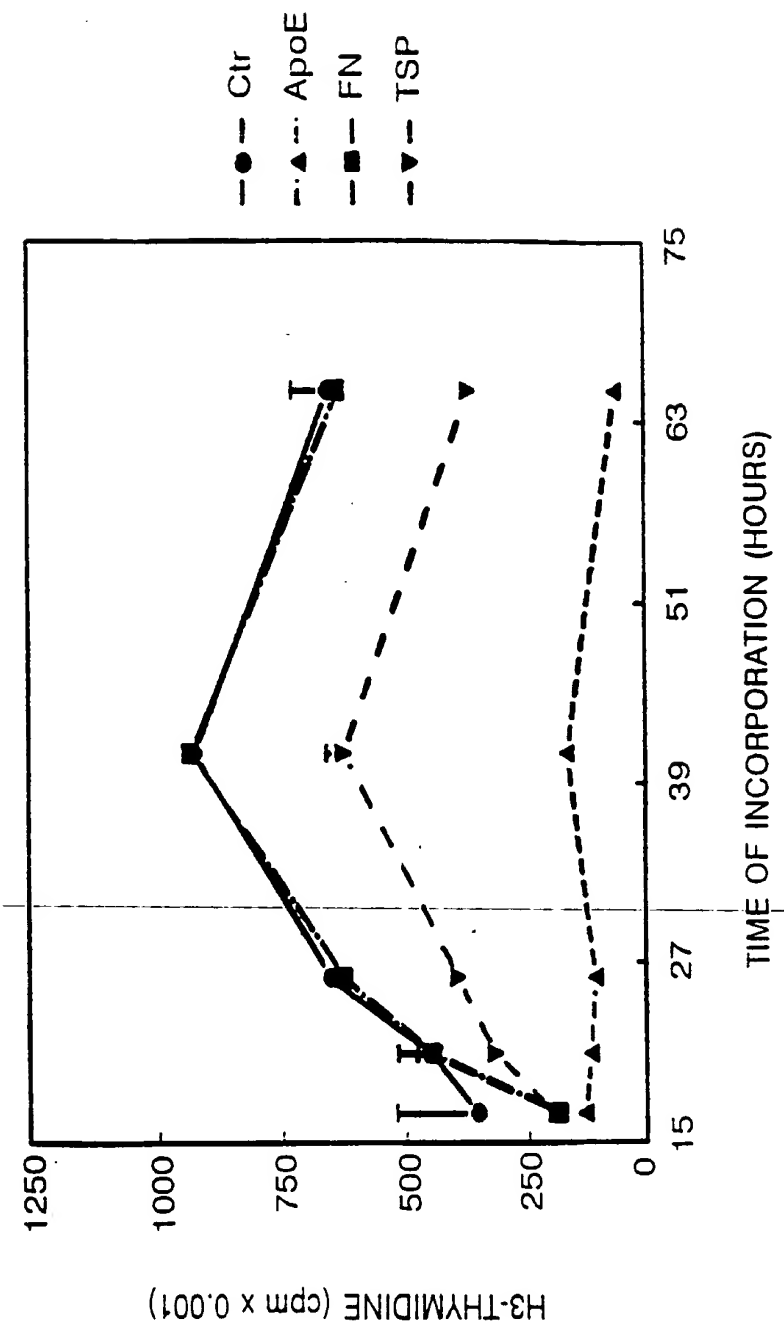

1% FCS
2.5% FCS
Ctr
bFGF
+E.05
+E.5
+T.05
+T.5
GROWTH EFFECTORS (μM)
0
32
64
96
128
160
H3-THYMIDINE (cpm x 0.001)

FIGURE 1

FIGURE 2


Ctr
ApoE
FN
TSP
1250
1000
750
500
250
0
15
27
39
51
63
75
TIME OF INCORPORATION (HOURS)
H3-THYMIDINE (cpm x 0.001)

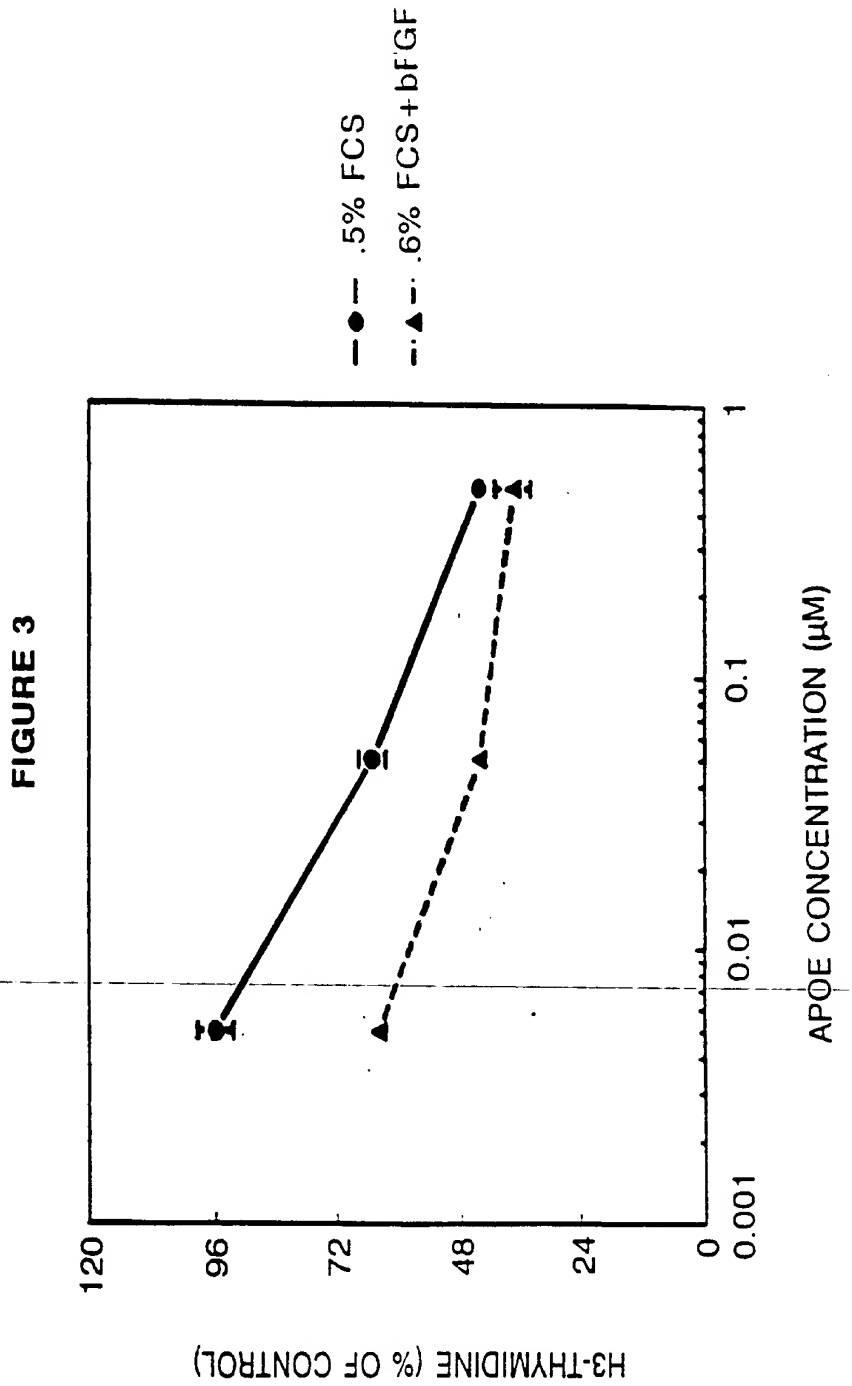
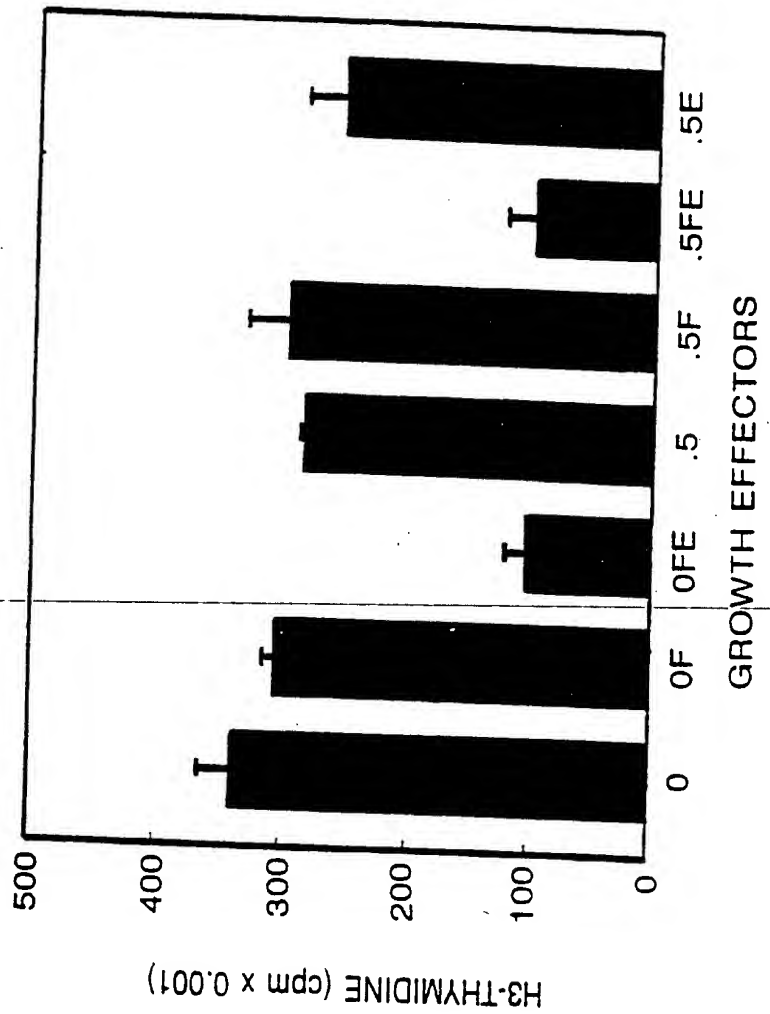

.5% FCS
.6% FCS+bFGF
APOE CONCENTRATION (μM)
0.001
0.01
0.1
1
H3-THYMIDINE (% OF CONTROL)
0
24
48
72
96
120

FIGURE 3

FIGURE 4


H3-THYMIDINE (cpm x 0.001)
500
400
300
200
100
0
0
0F
0FE
.5
.5F
.5FE
.5E
GROWTH EFFECTORS

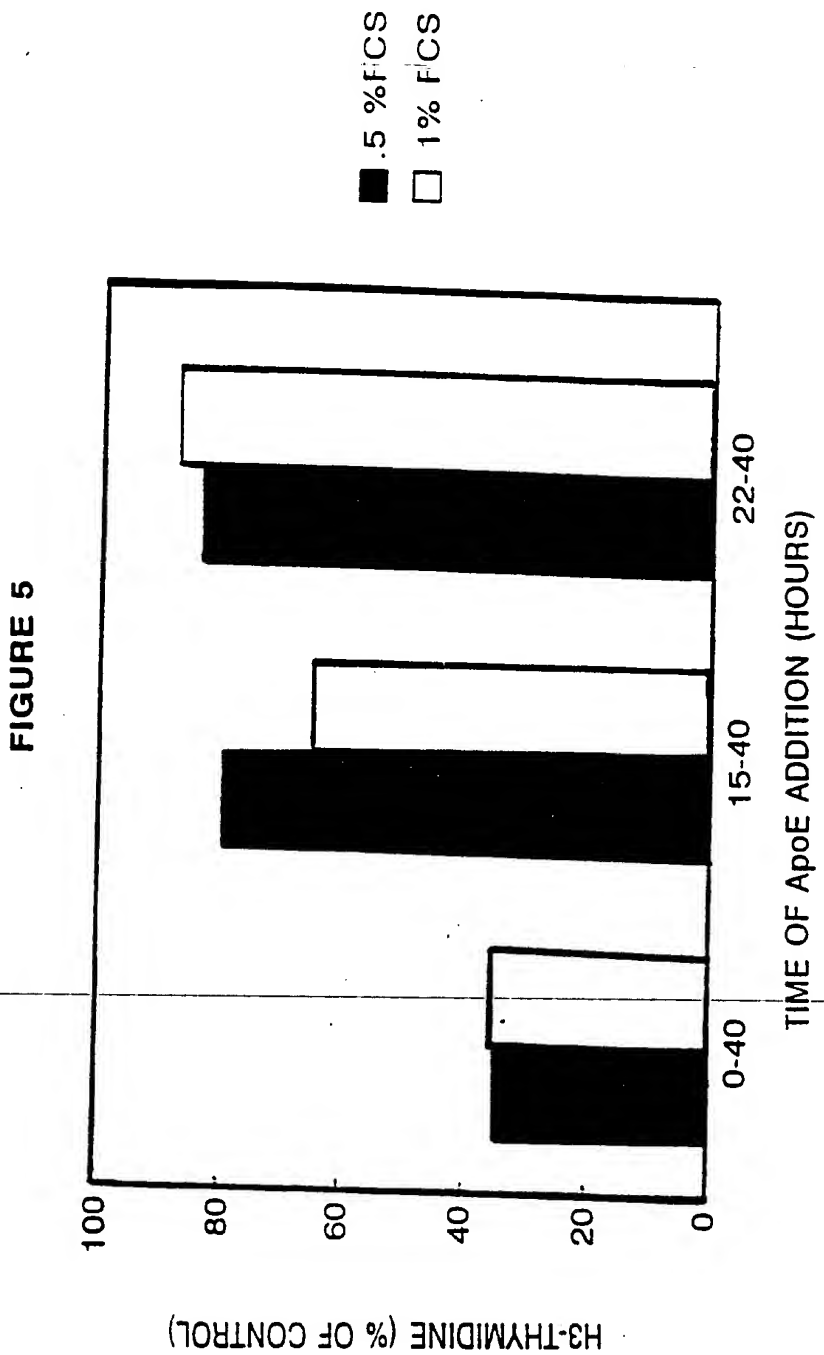

.5 %FCS
1% FCS
22-40
15-40
0-40
TIME OF ApoE ADDITION (HOURS)
100
80
60
40
20
0
H3-THYMIDINE (% OF CONTROL)

FIGURE 5

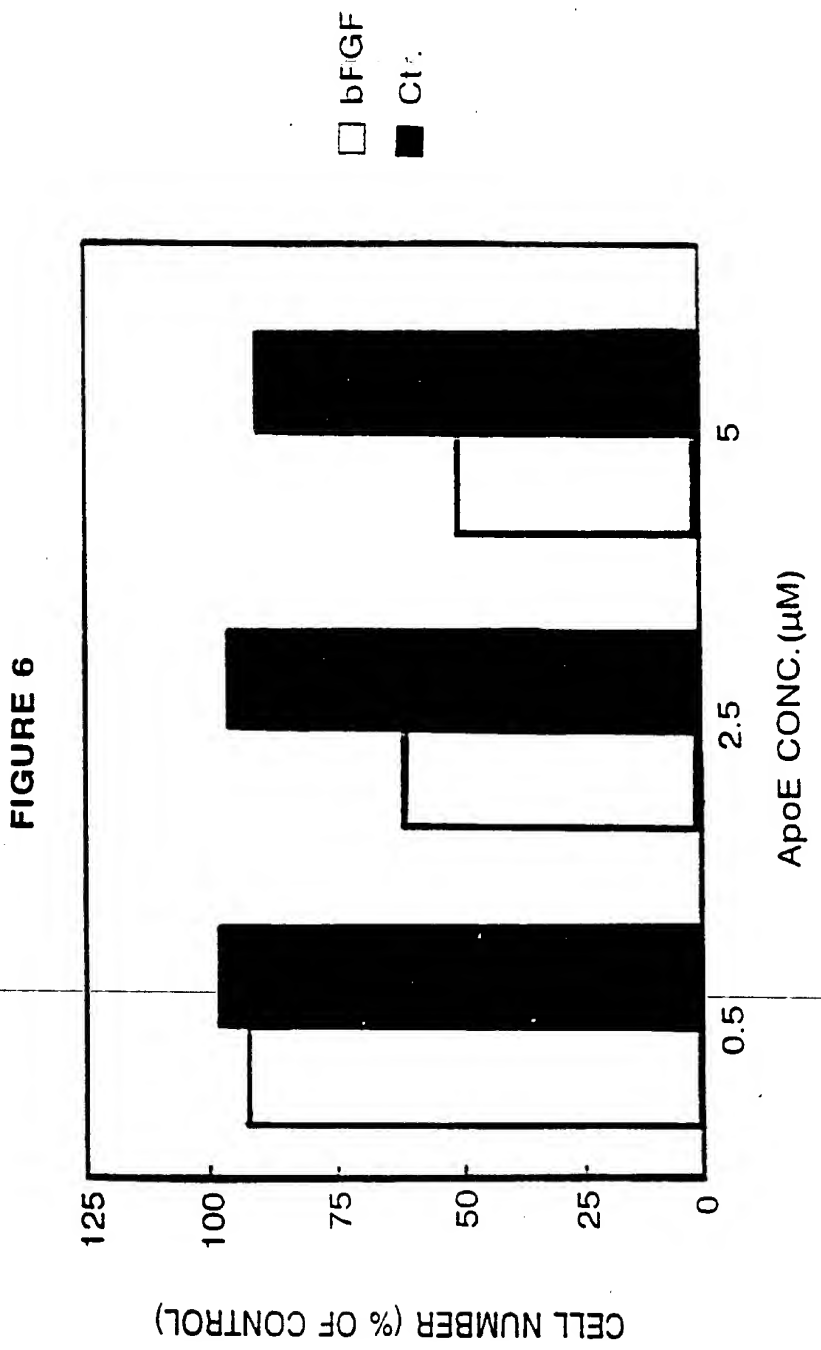

bFGF
Ctr.
ApoE CONC.(μM)
5
2.5
0.5
CELL NUMBER (% OF CONTROL)
125
100
75
50
25
0

FIGURE 6

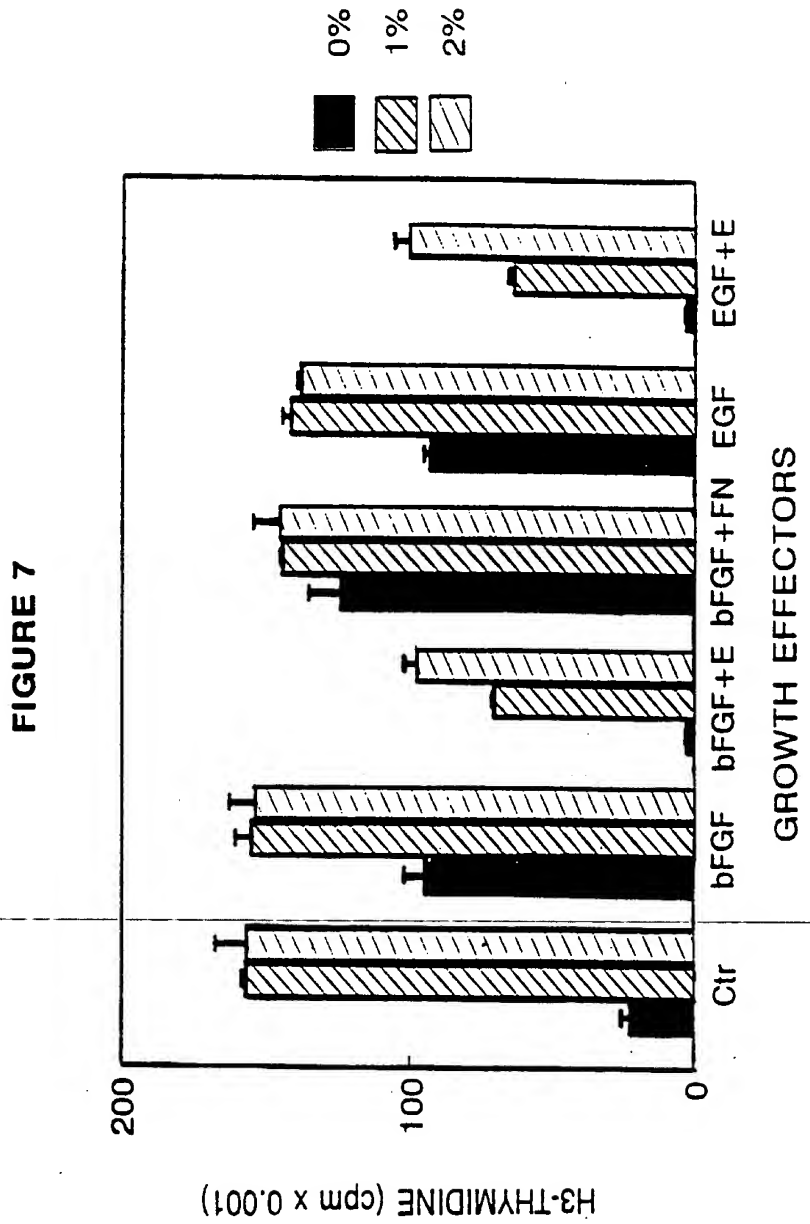

0%
1%
2%
200
100
0
H3-THYMIDINE (cpm x 0.001)
Ctr
bFGF
bFGF+E
bFGF+FN
EGF
EGF+E
GROWTH EFFECTORS

FIGURE 7

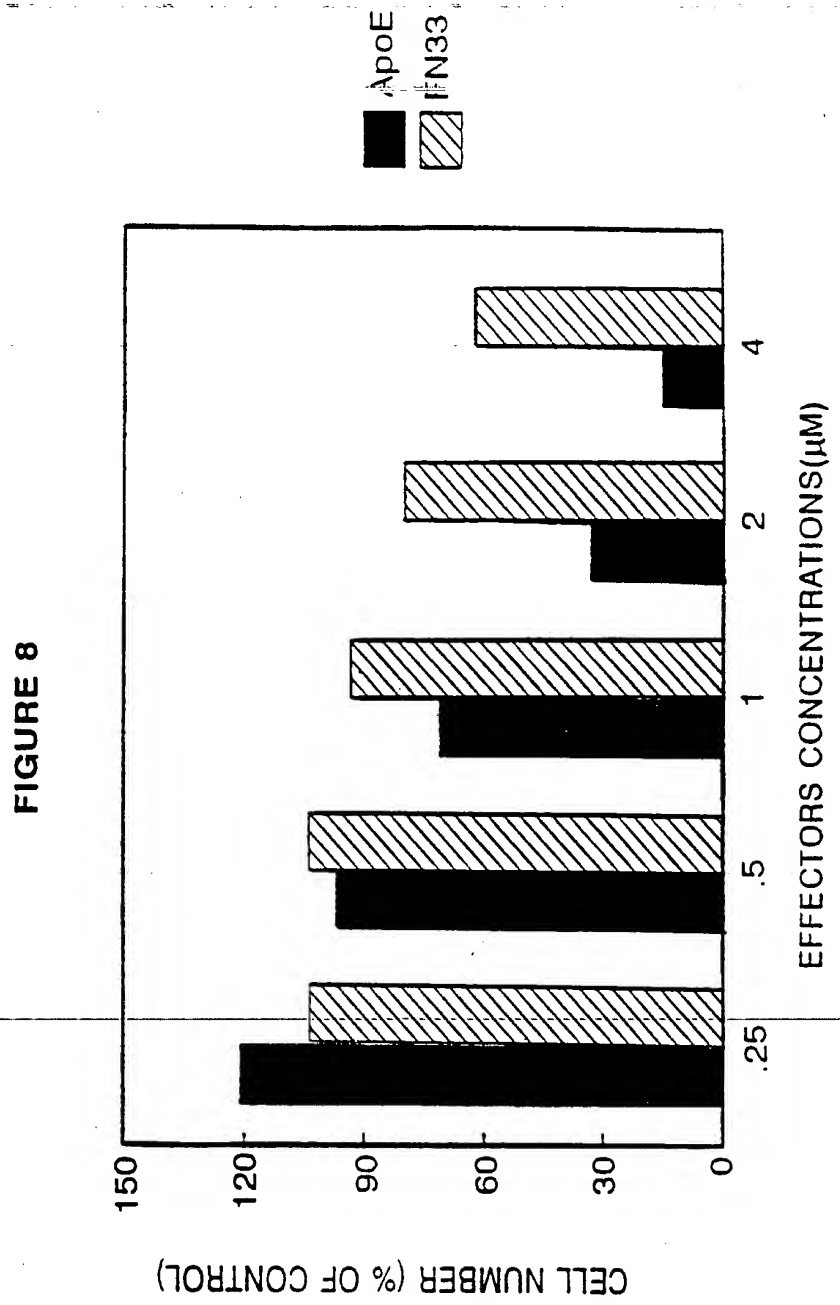

ApoE
N33
150
120
90
60
30
0
CELL NUMBER (% OF CONTROL)
.25
.5
1
2
4
EFFECTORS CONCENTRATIONS(μM)

FIGURE 8

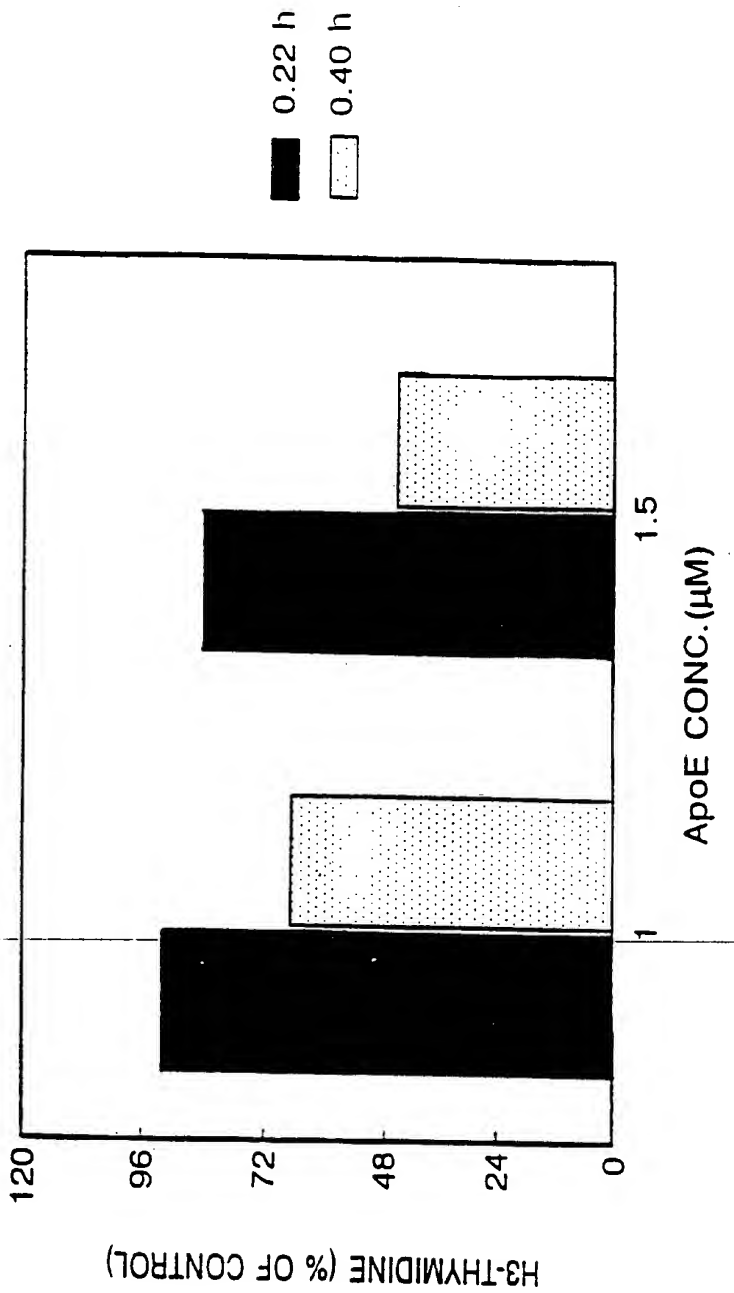
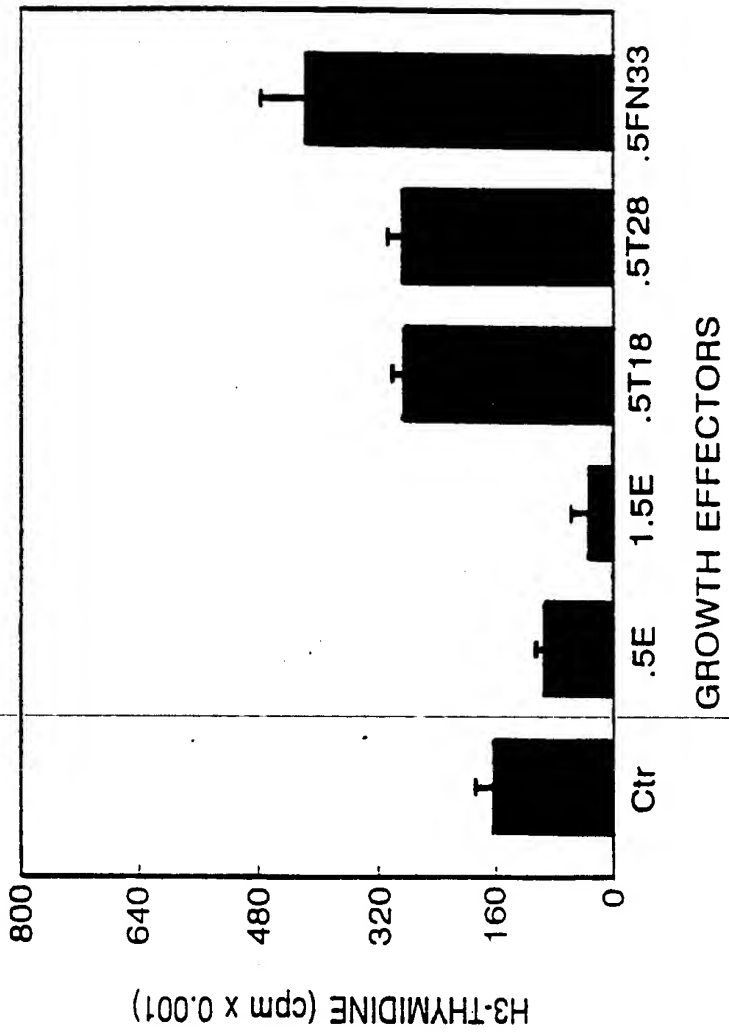

0.22 h
0.40 h
1.5
1
ApoE CONC.(μM)
120
96
72
48
24
0
H3-THYMIDINE (% OF CONTROL)

FIGURE 9

FIGURE 10


H3-THYMIDINE (cpm x 0.001)
800
640
480
320
160
0
Ctr
.5E
1.5E
.5T18
.5T28
.5FN33
GROWTH EFFECTORS

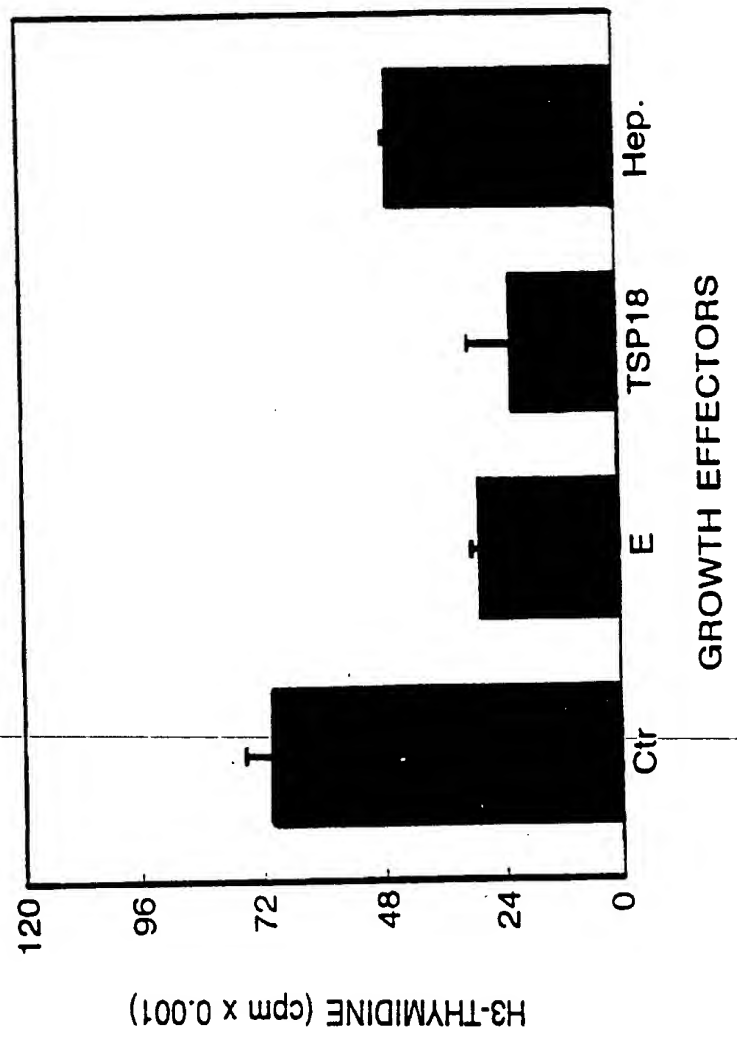

H3-THYMIDINE (cpm x 0.001)
120
96
72
48
24
0
Ctr
E
TSP18
Hep.
GROWTH EFFECTORS

FIGURE 11

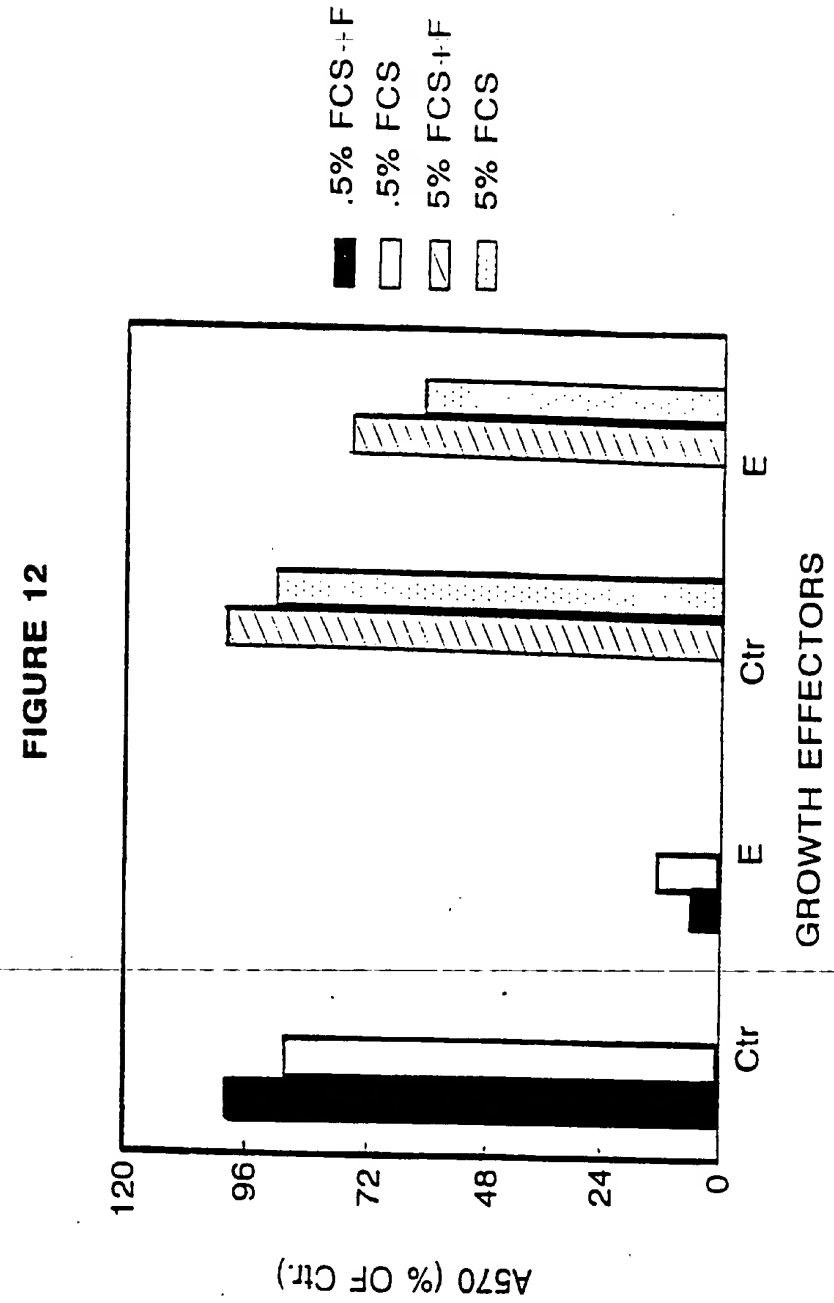
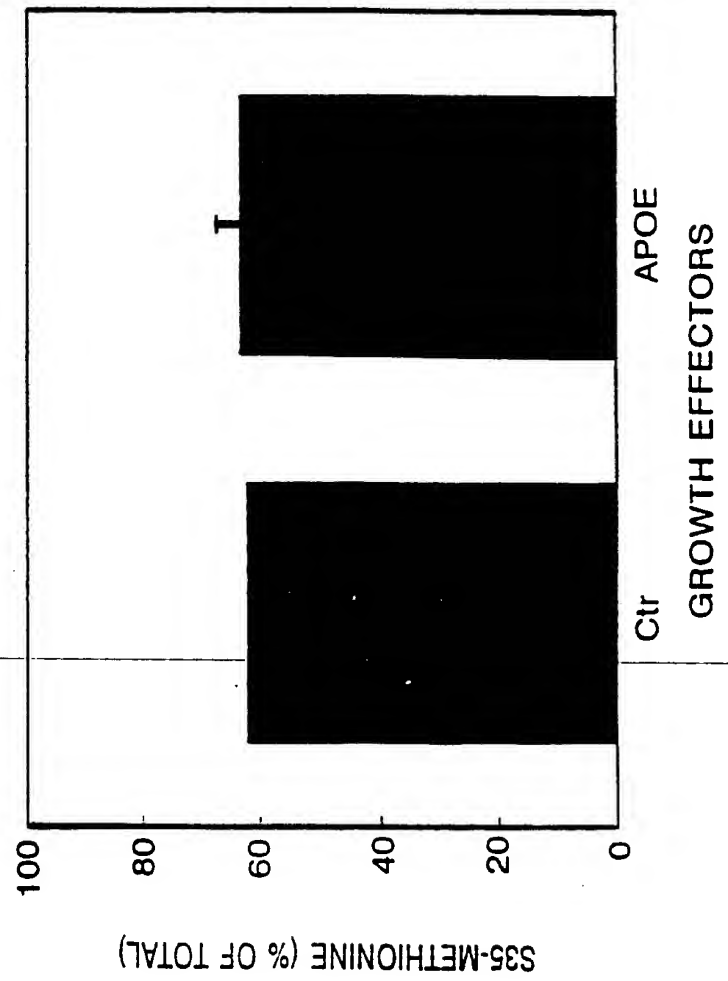

.5% FCS+F
.5% FCS
5% FCS+F
5% FCS
E
Ctr
E
Ctr
GROWTH EFFECTORS
A570 (% OF Ctr.)
120
96
72
48
24
0

FIGURE 12

FIGURE 13


GROWTH EFFECTORS
Ctr
APOE
S35-METHIONINE (% OF TOTAL)
0
20
40
60
80
100

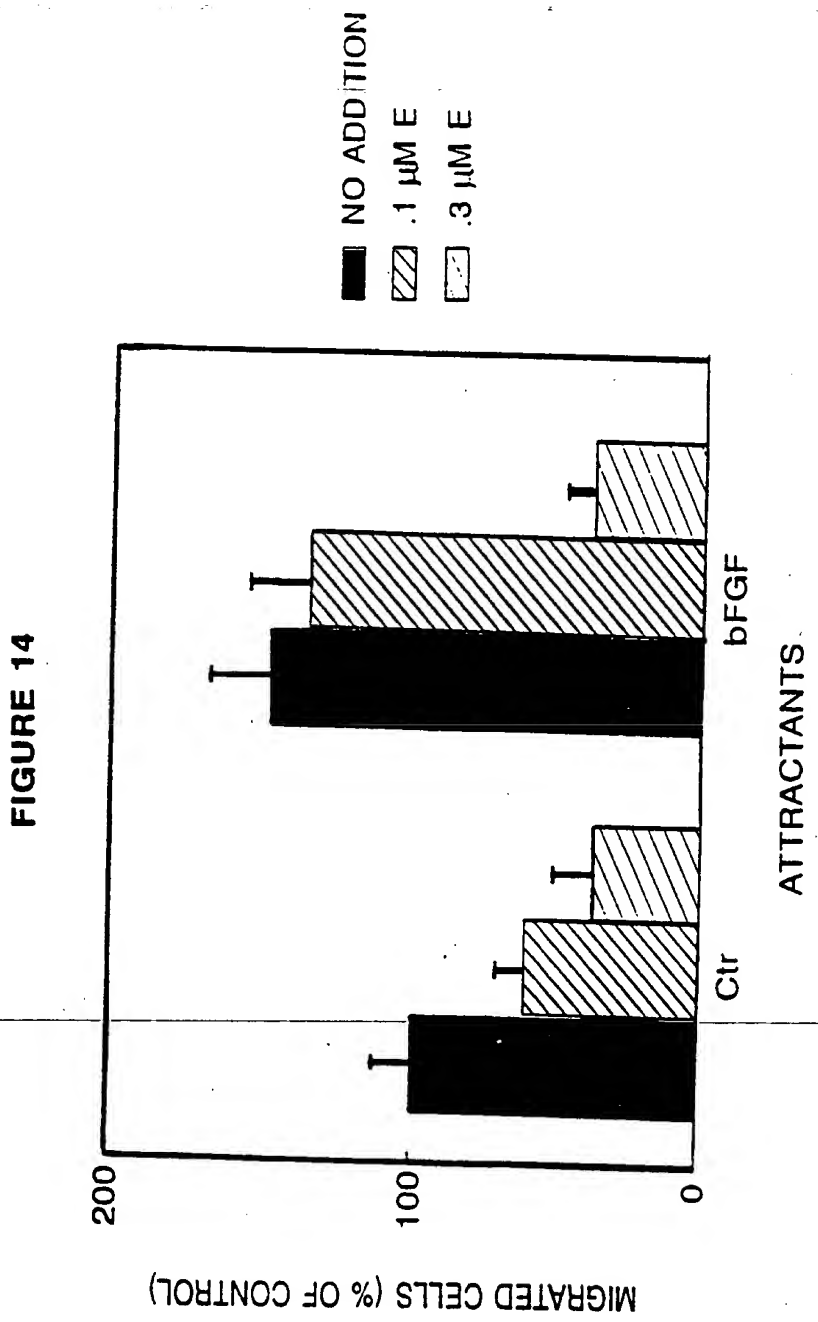

NO ADDITION
.1 μM E
.3 μM E
MIGRATED CELLS (% OF CONTROL)
200
100
0
Ctr
bFGF
ATTRACTANTS

FIGURE 14

FIGURE 15

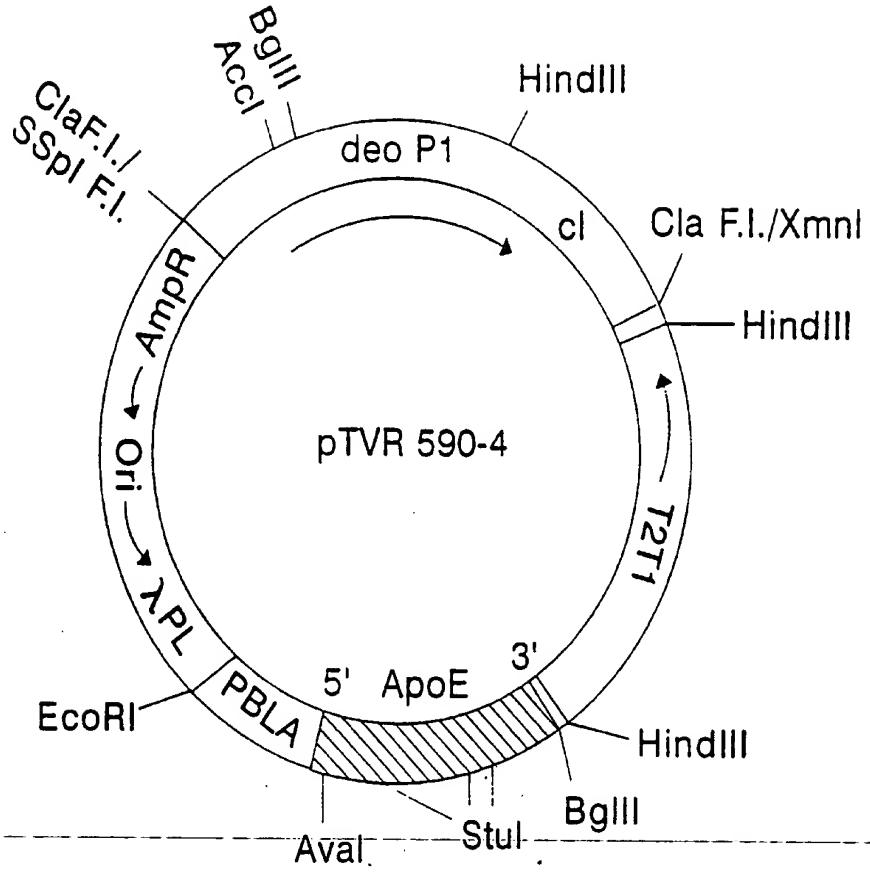
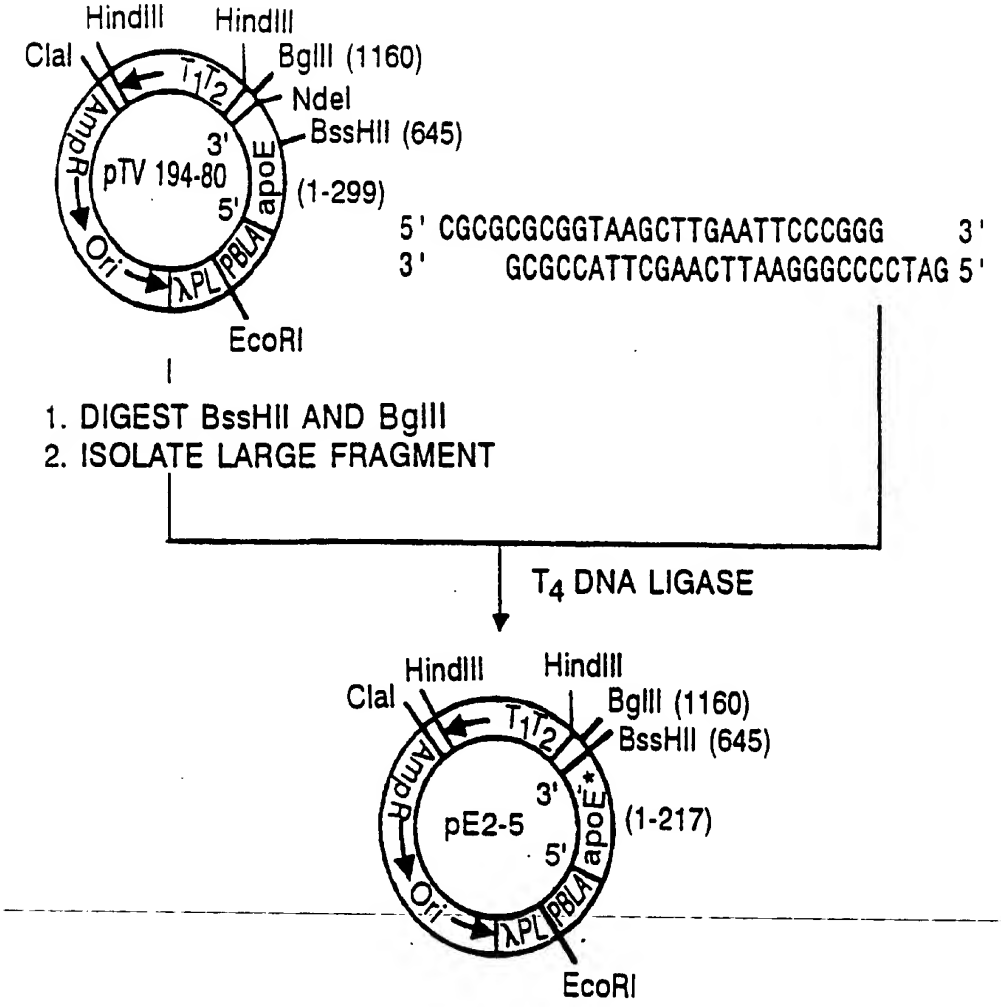

BglII
AccI
HindIII
ClaF.I./
SSpI F.I.
deo P1
cI
Cla F.I./XmnI
HindIII
AmpR
pTVR 590-4
Ori
λPL
T2T1
5'
ApoE
3'
PBLA
EcoRI
HindIII
BglII
AvaI
StuI

FIGURE 16


HindIII
HindIII
ClaI
BglII (1160)
NdeI
BssHII (645)
pTV 194-80
3'
5'
(1-299)
EcoRI
5' CGCGCGCGGTAAGCTTGAATTCCCGGG 3'
3' GCGCCATTCGAACTTAAGGGCCCCTAG 5'
1. DIGEST BssHII AND BglII
2. ISOLATE LARGE FRAGMENT
T4 DNA LIGASE
HindIII
HindIII
ClaI
BglII (1160)
BssHII (645)
pE2-5
3'
5'
(1-217)
EcoRI

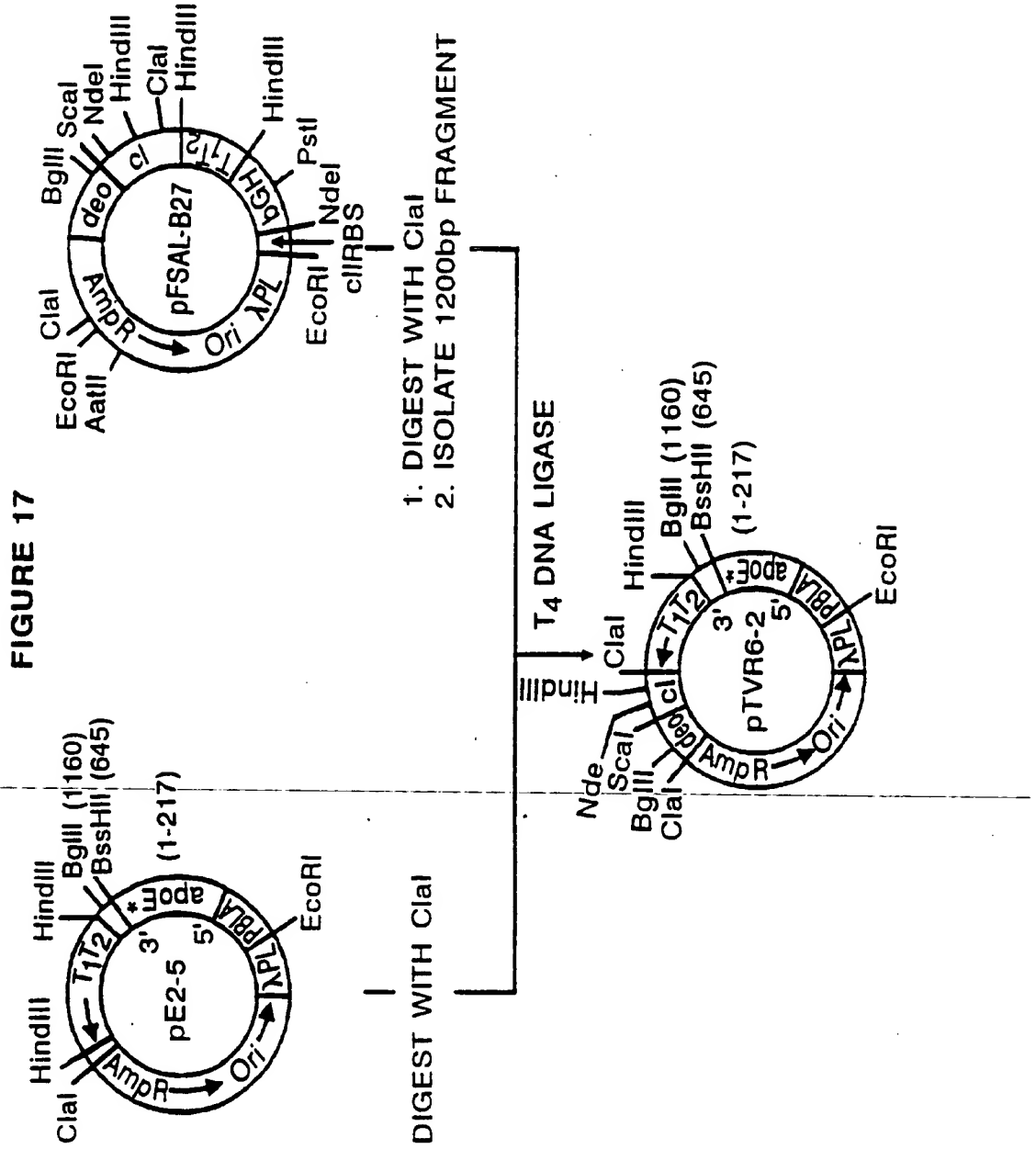
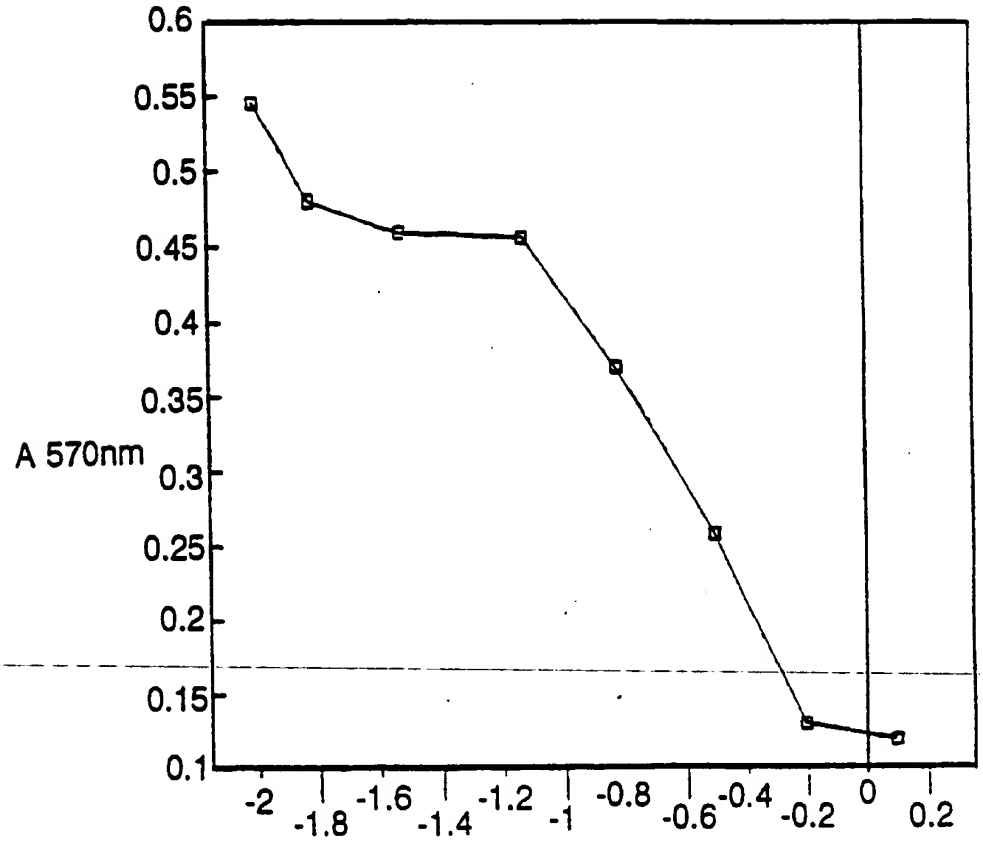

pFSAL-B27
Ori
λPL
AmpR
EcoRI
AatII
ClaI
deo
BglII
ScaI
NdeI
HindIII
cI
T1T2
ClaI
HindIII
HindIII
HGh
PstI
NdeI
EcoRI
cIIRBS
1. DIGEST WITH ClaI
2. ISOLATE 1200bp FRAGMENT
T4 DNA LIGASE
DIGEST WITH ClaI
pE2-5
Ori
AmpR
ClaI
HindIII
T1T2
3'
5'
apoE*
pBLA
λPL
EcoRI
HindIII
BglII (1160)
BssHII (645)
(1-217)
pTVR6-2
Ori
AmpR
deo
cI
T1T2
ClaI
HindIII
3'
5'
apoE*
pBLA
λPL
EcoRI
BglII (1160)
BssHII (645)
(1-217)
ScaI
NdeI
BglII
ClaI

FIGURE 17

FIGURE 18



18: ApoE (log μM)

FIGURE 19

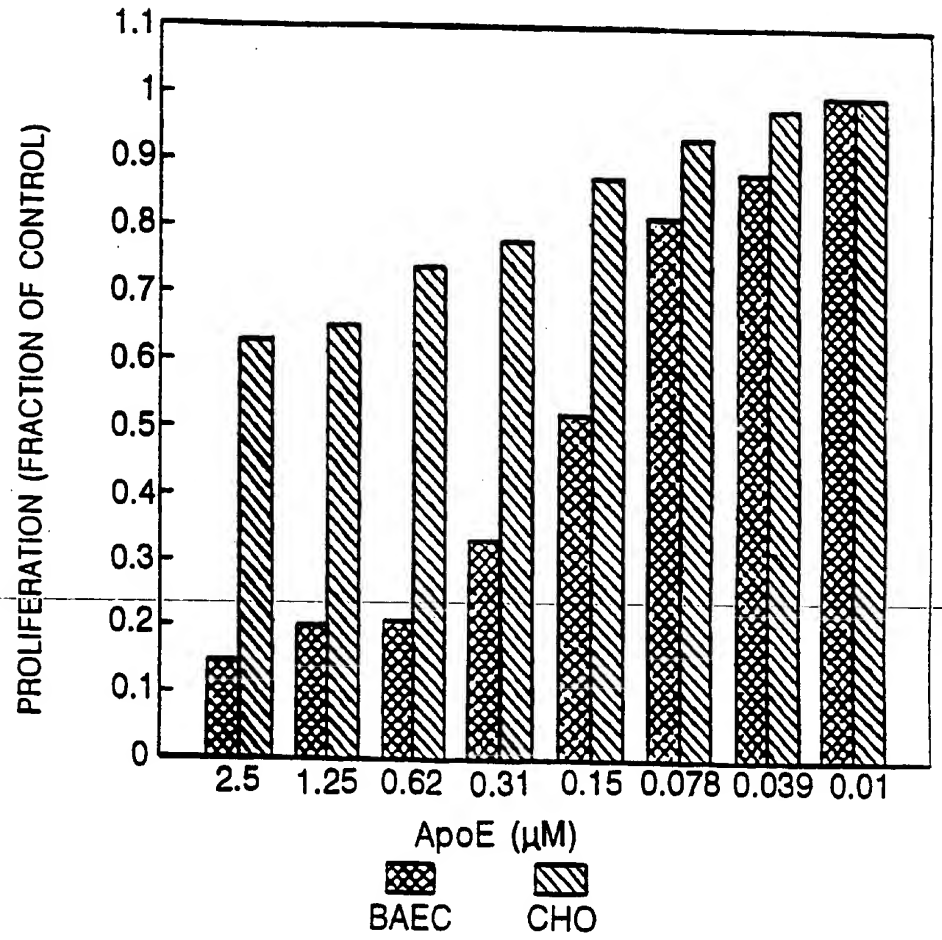

PROLIFERATION (FRACTION OF CONTROL)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2.5
1.25
0.62
0.31
0.15
0.078
0.039
0.01
ApoE (μM)
BAEC
CHO

FIGURE 20A

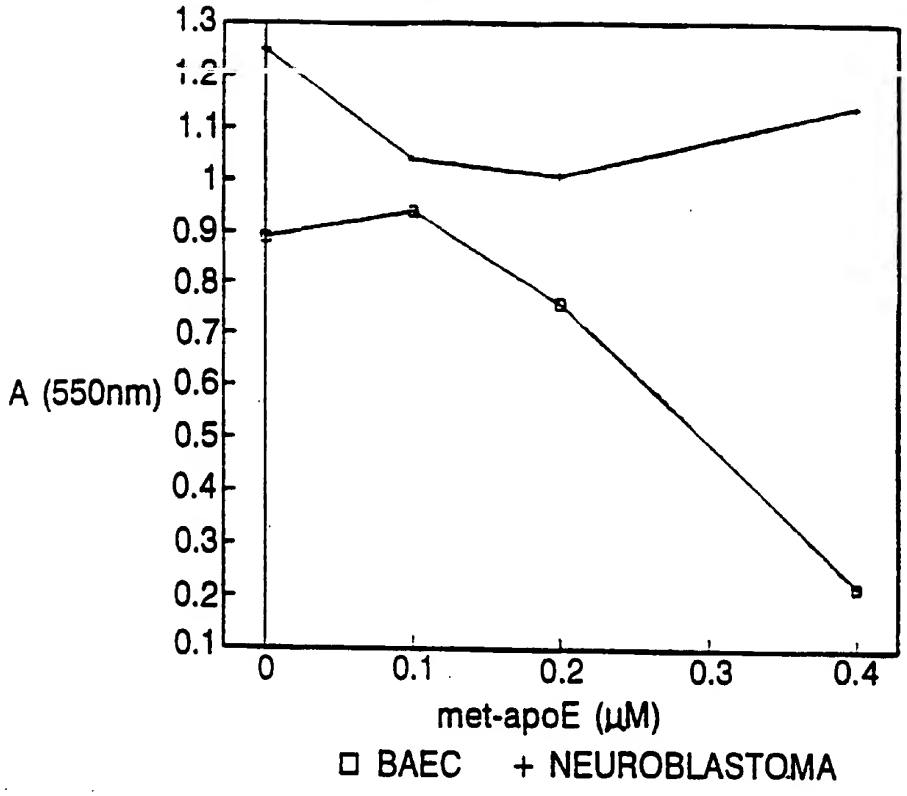


FIGURE 20B

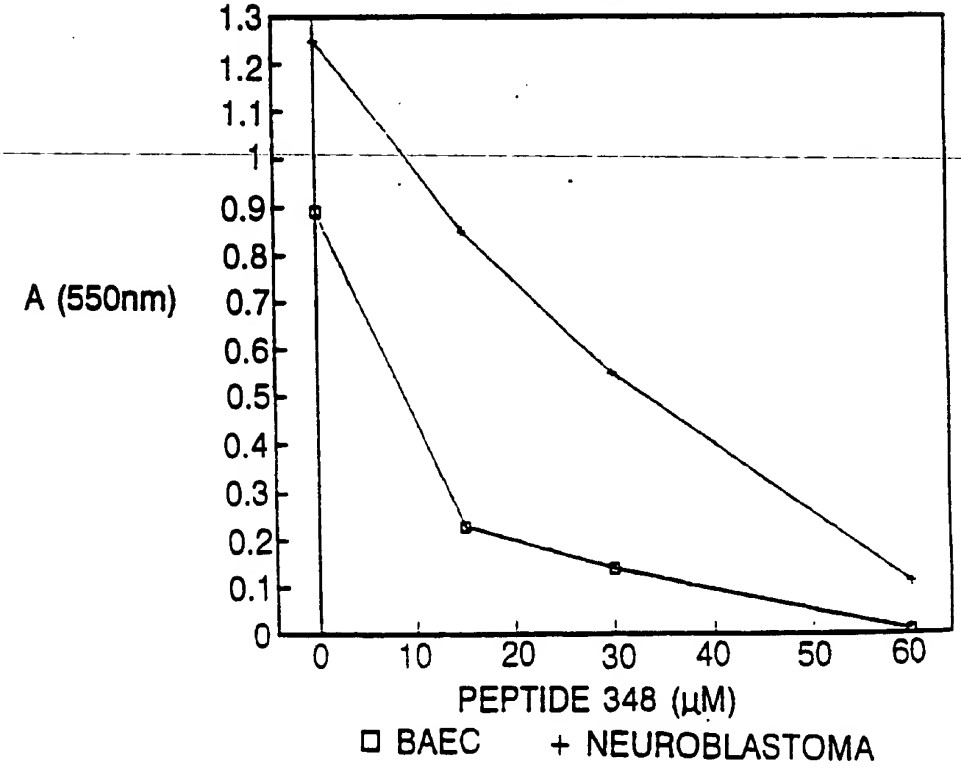


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FIGURE 21

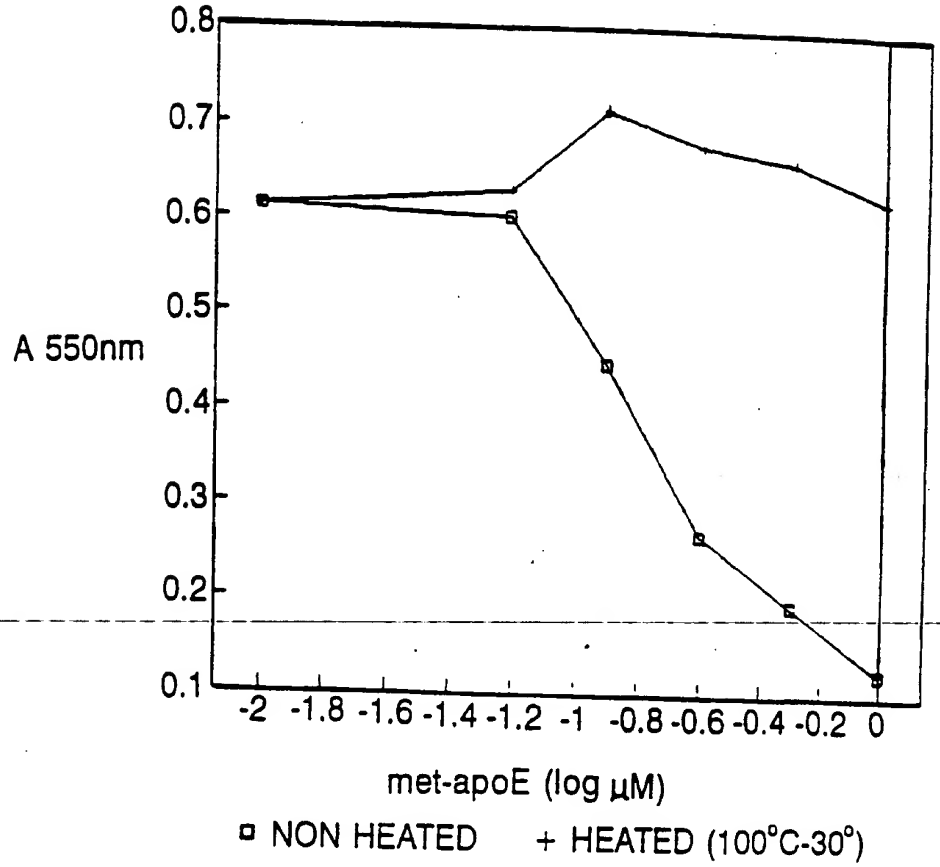


FIGURE 22

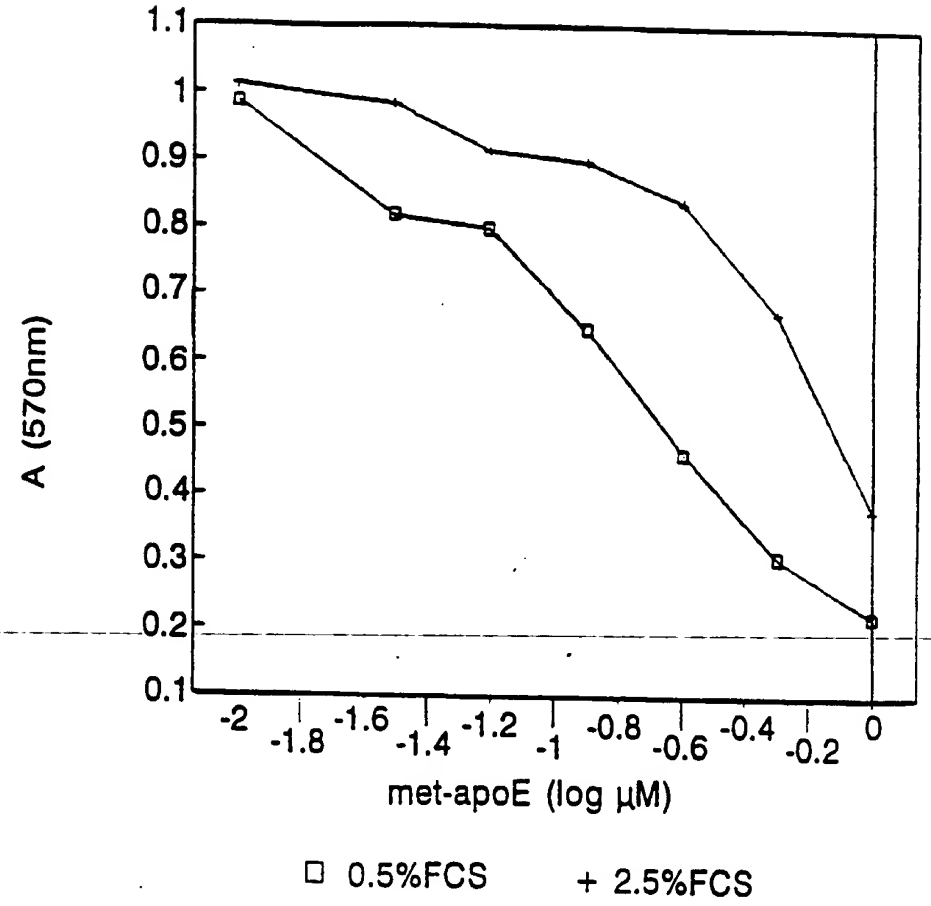

1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A (570nm)
-2
-1.8
-1.6
-1.4
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
met-apoE (log μM)
□ 0.5%FCS
+ 2.5%FCS

FIGURE 23

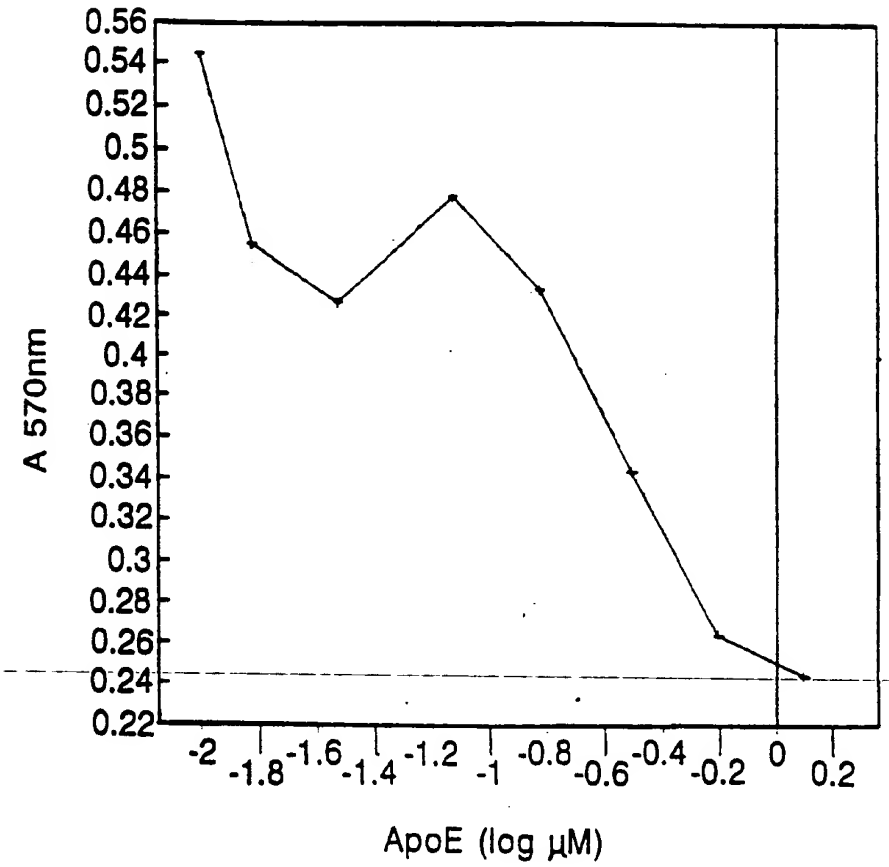

0.56
0.54
0.52
0.5
0.48
0.46
0.44
0.42
0.4
0.38
0.36
0.34
0.32
0.3
0.28
0.26
0.24
0.22
A 570nm
-2
-1.8
-1.6
-1.4
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
ApoE (log µM)

FIGURE 24

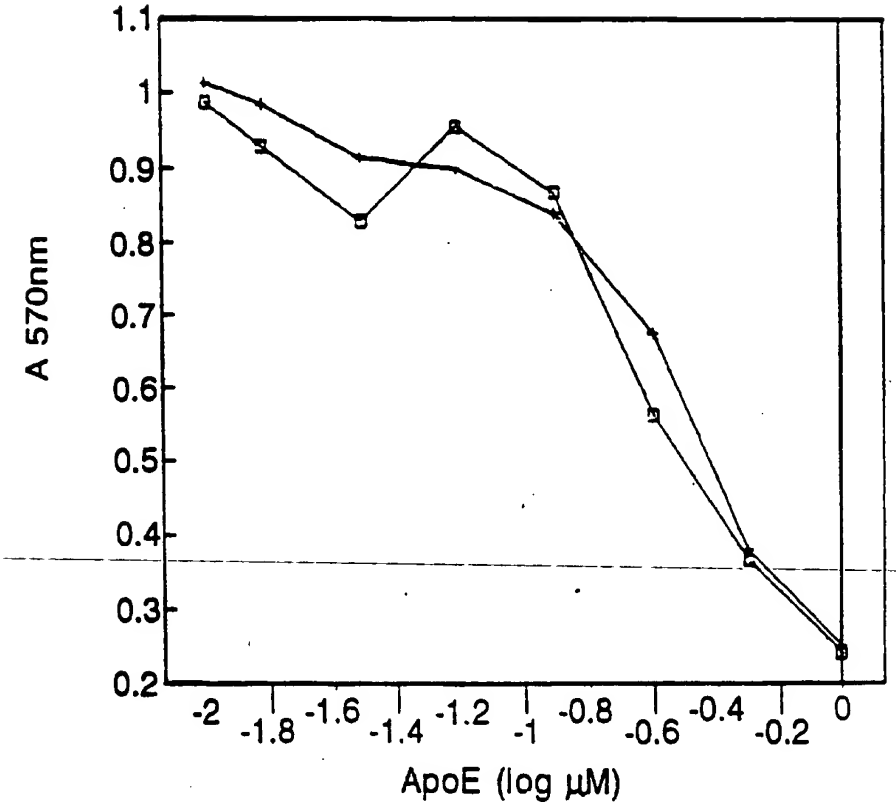
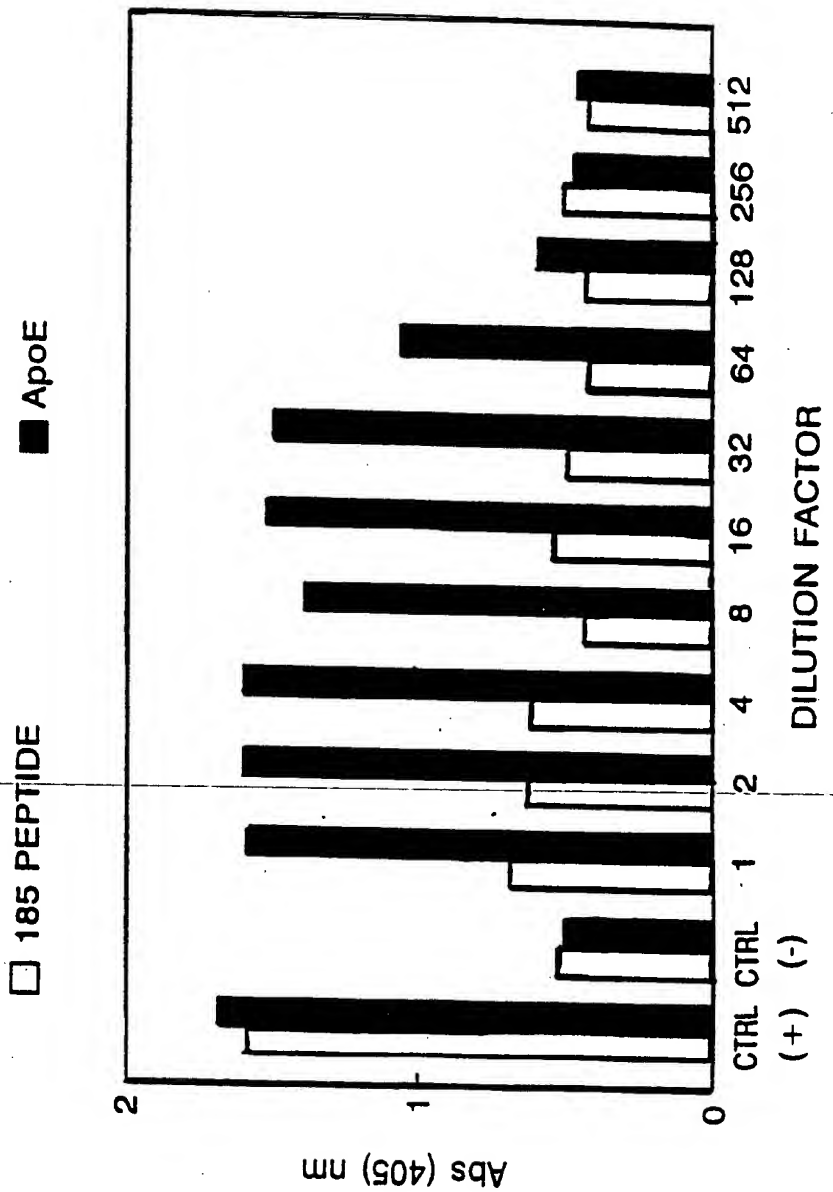

1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
A 570nm
-2
-1.8
-1.6
-1.4
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
ApoE (log µM)
□ 0.5%FCS
+ 2.5%FCS

FIGURE 25


ApoE
185 PEPTIDE
Abs (405) nm
0
1
2
CTRL (+)
CTRL (-)
1
2
4
8
16
32
64
128
256
512
DILUTION FACTOR

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US93/07582

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 37/02, 37/22
US CL :514/12, 530/380
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/12, 530/380

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Dialog: Medline, Biosis Previews, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Science, Volume 240, issued 29 April 1988, R. W. Mahley, "Apolipoprotein E: Cholesterol Transport Protein with Expanding Role in Cell Biology", pages 622-630, entire document	1-28
A	Biochemical and Biophysical Research Communications, Volume 154, No. 2, issued 29 July 1988, A. D. Cardin et al, "Inhibition of Lymphocyte Proliferation by Synthetic Peptides Homologous to Human Plasma Apolipoproteins B and E", pages 741-745, entire document.	1-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1993	

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MICHAEL WITYSHYN
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